# CARBOXYLESTERASE 1 GENETIC VARIABILITY, EXPRESSION AND POTENTIAL FOR DRUG-DRUG INTERACTIONS

Thesis submitted in accordance with the requirements of the University of Liverpool for the degree of Doctor in Philosophy

By

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# ABSTRACT

Carboxylesterase 1 (CES1) is the main human liver esterase and is involved in the metabolism and disposition of numerous endogenous and pharmacological compounds. Some of the substrates of this enzyme are widely prescribed agents such as clopidogrel (Plavix®), methylphenidate (Ritalin®) and oseltamivir (Tamiflu®). However, there is much uncertainty regarding the genetic variability within CES1, and its regulation and involvement in drug-drug interactions (DDI).

Polypharmacy is frequent in elderly, HIV and tuberculosis infected populations, and the risk of harmful DDIs is high, especially when these populations overlap. The role played by CES1 on the treatment of all these three pathologies and vice versa needs to be better characterized. In this thesis the role of CES1 genetic variability and its potential role in DDIs are explored both in isolation and in conjunction with other genetic, demographic, physio-pathological and iatrogenic factors.

The impact of CES1 genetic variability was assessed on the anti-platelet effect of clopidogrel as well as on isoniazid pharmacokinetics in acute coronary syndrome (ACS) and HIV/Tuberculosis co-infected populations respectively. DDIs mediated by CES1 were explored in a HIV positive cohort treated with clopidogrel and non-nucleoside reverse transcriptase inhibitors (NNRTIs). Also, *in vitro* experiments with primary hepatocytes were used to investigate CES1 intracellular expression in the presence of prototypical PXR inducers used in tuberculosis treatment.

The results of this thesis show that the CES1 rs2244613 SNP does affect clopidogrel anti-aggregant activity and may contribute to treatment non-response. Another CES1 variant, rs3815583, was found to be associated with changes in isoniazid pharmacokinetics. The studies did not indicate that NNRTI co-administration with clopidogrel would impair the anti-platelet activity since no relevant changes in exposure of the antiplatelet agent were identified. In the same way, the results do not anticipate DDIs between CES1 substrates and rifamycins, since no induction of expression was identified after incubating primary human hepatocytes *in vitro* with rifampicin, rifabutin and rifapentine.

In conclusion, the results shown in this thesis support the idea that CES1 genetic variability may play a bigger role than previously suspected in treatment response but may not be a mediator of clinically relevant DDIs.

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# ABBREVIATIONS

| | |
|---|---|
| µg | Microgram(s) |
| µl | Microliter(s) |
| µM | Micromolar |
| µm | Micrometre(s) |
| µmol | Micromole |
| A | Adenine |
| ABC | ATP binding cassette |
| ACAT | Acyl-coenzymeA:cholesterol acyltransferase |
| ACEI | Angiotensin converting enzyme inhibitor |
| ACN | Acetonitrile |
| ACS | Acute coronary syndrome |
| ADP | Adenosine diphosphate |
| ANOVA | Analysis of variance |
| ARE | Antioxidant response element |
| ART | Antiretroviral therapy |
| ARV | Antiretroviral |
| AST | Aspartate transaminase |
| ATP | Adenosine triphosphate |
| AUC | Area under the curve |
| BMI | Body mass index |
| bp | Base pair |
| C | Cytosine |
| CABG | Coronary artery bypass grafting |
| CAR | Constitutive androstane receptor |
| cDNA | Coding DNA |
| CEH | Cholesteryl ester hydrolase |
| CES1 | Carboxylesterase 1 |
| CES1A1 | Carboxylesterase 1A1 gene isoform |
| CES1A2 | Carboxylesterase 1A2 gene isoform |
| CES1A3 | Carboxylesterase 1A3 pseudogene |
| CES2 | Carboxylesterase 2 |
| CES4 | Carboxylesterase 4 |
| CHRM | Cryopreserved hepatocyte recovery medium |
| CLP | Clopidogrel |
| CLPM | Clopidogrel inactive metabolite |
| $C_{max}$ | Maximum concentration |
| CNV | Copy number variation |

| | |
|---|---|
| CRP | C-reactive protein |
| $C_T$ | Cycle threshold |
| CV | Coefficient of variation |
| CYP P450 | Cytochrome P450 |
| DDI | Drug-drug interactions |
| DEX | Dexamethasone |
| dl | Decilitre(s) |
| DNA | Deoxyribonucleic acid |
| E. coli | Escherichia coli |
| ECG | Electrocardiogram |
| EDTA | Ethylamine-diaminetetra-acetic acid |
| EFV | Efavirenz |
| EI | Entry inhibitors |
| EMB | Ethambutol |
| ER | Endoplasmic reticulum |
| FAEE | Fatty acid ethyl ester |
| FAM | 6-carboxyfluorescein |
| FDA | Food and drug administration |
| g | Gram(s) |
| G | Guanine |
| GABA | Gamma-aminobutyric acid |
| GAPDH | Glyceraldehyde 3-phosphate dehydrogenase |
| gDNA | Genomic DNA |
| GR | Glucocorticoid receptor |
| h | Hour(s) |
| $H_2O$ | Water |
| HAART | Highly active antiretroviral therapy |
| Hb | Haemoglobin |
| HCV | Hepatitis C virus |
| HIV | Human immunodeficiency virus |
| HMSE | Human monocyte/macrophage serine esterase |
| HPLC | High performance liquid chromatography |
| HQC | High quality control |
| i.u. | International units |
| IL-6 | Interleukin 6 |
| Inc | Incorporated |
| INH | Isoniazid |
| InI | Integrase inhibitor |
| IQR | Inter quartile range |
| IS | Internal standard |

| | |
|---|---|
| kbp | Kilobase pair |
| kg | Kilogram(s) |
| $KH_2PO_4$ | Monopotassium phosphate |
| L | Litre(s) |
| LC-MS | Liquid chromatography tandem mass spectrometry |
| LD | Linkage disequilibrium |
| LLOQ | Lower limit of quantitation |
| LOD | Limit of detection |
| LPS | Lipopolysaccharide |
| LQC | Low quality control |
| LTA | Light transmission aggregometry |
| Ltd | Limited |
| M | Molar |
| MAF | Minor allele frequency |
| MDR-TB | Multidrug resistant tuberculosis |
| mg | Milligram(s) |
| min | Minutes |
| ml | Millilitre(s) |
| mM | Millimolar |
| mm | Millimetre |
| MPH | Methylphenidate |
| MQC | Medium quality control |
| mRNA | Messenger RNA |
| MS | Mass Spectrometry |
| n | Number |
| N | Total sample size |
| N/A | Not applicable |
| NA | Nucleoside analogue |
| NADPH | Nicotinamide adenine dinucleotide phosphate |
| NAT2 | N-acetyltransferase |
| ng | Nanogram(s) |
| nm | Nanometre(s) |
| nM | Nanomolar |
| NNRTI | Non-nucleoside reverse transcriptase inhibitor |
| NRF2 | Nuclear factor erythroid 2 |
| NSTEMI | Non-ST segment elevation myocardial infarction |
| NVP | Nevirapine |
| OAT | Organic anion transporter |
| $^oC$ | Degree Celsius |
| OCT | Organic cation transporter |

| | |
|---|---|
| OFA | Orthophosphoric acid |
| PCI | Percutaneous coronary intervention |
| PCR | Polymerase chain reaction |
| pg | Picogram(s) |
| P-gp | P-glycoprotein |
| PhACS | Pharmacogenomics of acute coronary syndrome |
| PhD | Doctor of Philosophy |
| PHE | Phenobarbital |
| PI | Protease inhibitor |
| PK | Pharmacokinetic |
| PXR | Pregnane X receptor |
| PZA | Pyrazinamide |
| QC | Quality control |
| qPCR | Real-time polymerase chain reaction |
| Q-Q | Quantile-quantile |
| RBT | Rifabutin |
| REH | Retinyl ester hydrolase |
| RFP | Rifapentine |
| RIF | Rifampicin |
| RNA | Ribonucleic acid |
| rpm | Revolutions per minute |
| RT-PCR | Reverse transcriptase polymerase chain reaction |
| s | Second(s) |
| SD | Standard deviation |
| SDM | Site-directed mutagenesis |
| SLC | Solute carriers |
| SNP | Single nucleotide polymorphism |
| STEMI | ST segment elevation myocardial infarction |
| T | Thymine |
| $t_{1/2}$ | Half-life |
| TB | Tuberculosis |
| TDM | Therapeutic drug monitoring |
| TGH | Triacylglycerol ester hydrolase |
| $T_{max}$ | Time of maximum concentration |
| TNF-α | Tumor necrosis factor alpha |
| UK | United Kingdom |
| USA | United States of America |
| UTR-5' | Five prime untranslated region |
| UV | Ultraviolet |
| v/v | Volume to volume ratio |

| | |
|---|---|
| VIC | 4,7,2′-trichloro-7′-phenyl-6-carboxyfluorescein |
| w/vol | weigth/volume |
| WT | Wild-type |
| XDR-TB | Extensively drug resistant tuberculosis |
| $\lambda_z$ | Lambda z |

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## Table of Single Nucleotide Polymorphisms (SNPs) assessed in this PhD thesis

| Accession Number | Gene | Chromosome | Haplotype | Genomic location | Nucleotide change | Aminoacid change |
|---|---|---|---|---|---|---|
| rs71647871 | CES1 | 16 | N/A | Exon 4 | G>A | p.G143E |
| rs71647872 | CES1 | 16 | N/A | Exon 6 | T>- | p.R260- |
| rs62028647 | CES1 | 16 | N/A | Exon 2 | G>A | p.S83L |
| rs3826190 | CES1 | 16 | N/A | Exon 2 | G>T | p.G18V |
| rs2244613 | CES1 | 16 | N/A | Intronic | A>C | N/A |
| rs3815583 | CES1 | 16 | N/A | 5'UTR region | T>G | N/A |
| rs1799853 | CYP2C9 | 10 | CYP2C9 (*2) | Exon 3 | C>T | p.R144C |
| rs1057910 | CYP2C9 | 10 | CYP2C9 (*3) | Exon 7 | A>C | p.I359L |
| rs4244285 | CYP2C19 | 10 | CYP2C19 (*2) | Exon 5 | G>A | p.P227P |
| rs12248560 | CYP2C19 | 10 | CYP2C19 (*17) | 5'UTR region | C>T | N/A |

# CHAPTER 1

# General introduction

# Chapter 1: General Introduction

## 1.1. Human metabolism

### 1.1.1. Drug metabolising enzymes

Following absorption, therapeutic agents undergo a series of reactions, such as oxidation, reduction or conjugation intended to clear the body of what it considers to be an external toxicant. These reactions are catalysed by metabolic enzymes that are present in different tissues in the body, but predominantly in the liver and gastrointestinal tract (Krishna and Klotz 1994). Metabolism is divided in three phases; phase I, phase II and phase III.

Oxidation and reduction are carried out mainly by cytochrome P450 enzymes (Zollner et al. 2010), a group of hemeproteins containing a metal ion at their core that readily interconvert between the reduced and oxidized state and which require the presence of NADPH to function. Cytochrome P450s are the major enzymes in drug metabolism carrying out about 75% of metabolic reactions (Wang and Chou 2010). They are classified in 4 main families: CYP1, CYP2, CYP3 and CYP4, and these in numerous subfamilies with different affinity for substrates. CYP3A4 is the most abundant CYP enzyme and catalyses about 50% of all therapeutic compounds on the market. Other key CYP enzymes relevant to drug metabolism and some of their substrates are shown in Table 1-1.

Hydrolysis is the main reaction that esters and amides undergo as part of phase I metabolism. Esters are generally hydrolysed by esterases and amides by amidases. Amides can be metabolized by esterases, but at a much slower rate. There are non-specific esterases present in plasma and more specific enzymes present mainly in the liver.

Esterases are ubiquitously expressed and there are numerous isoenzymes of esterases with broad substrate overlap, they process about 10% of therapeutic compounds on the market (Fukami and Yokoi 2012).

The esterification of a carboxylic acid functional group is a frequently used strategy to generate prodrugs with increased lipophilicity and thereby enhanced absorption rates. It is possible to prepare ester derivatives with virtually any degree of lipophilicity that will release the active drug quickly after absorption due to the action of esterases.

Phase II reactions are conjugation reactions and include glucuronidation, sulfation, methylation, acetylation, amino acid conjugation, glutathione conjugation and fatty acid conjugation (Jancova et al. 2010).

Phase III refers to the action of transporters that help drugs and metabolites to cross lipid cell membranes. For example, in the liver transporters move drug conjugates into the bile, in the kidney they transport xenobiotics from blood into the urine.

**Table 1-1 Main CYP enzymes involved in drug metabolism in humans.**

| CYP | Substrates |
|---|---|
| CYP1A2 | Caffeine, NSAIDs, theophylline, warfarin |
| CYP2A6 | Nicotine, coumarine |
| CYP2B6 | Efavirenz, bupropion, cyclophosphamide, methadone, nevirapine |
| CYP2C8 | Pioglitazone, cerivastatin, ibuprofen, paclitaxel |
| CYP2C9 | Warfarin, phenytoin, etravirine |
| CYP2C19 | Clopidogrel, omeprazole, NSAIDs, nelfinavir |
| CYP2D6 | Fluoxetine, tamoxifen, vincristine, haloperidol, quinidine |
| CYP3A4 | Midazolam, HIV protease inhibitors, benzodiazepines, macrolides |
| CYP3A5 | Nifedipine, cyclosporine, saquinavir |

### 1.1.2. Drug transporters

Drug transporters are trans-membranous proteins that are embedded in intra- or extracellular membranes. They assist drugs and endogenous substances in moving between compartments against a concentration gradient in an ATP-dependent process (Leuthold et al. 2009).

There are two main types of drug transporters, ATP binding cassette (ABC) proteins that are generally involved in molecule efflux, and solute carriers (SLC) which usually participate in the influx of molecules (Dean et al. 2001). The latter can be divided into OATP and OCTP, organic anion/cation transporter proteins. Some of the main drug transporters are shown in Table 1-2.

Drug transporters display considerable variability in expression and activity, as they are susceptible to modulation by endogenous and exogenous substances. There is also differential expression of transporters between tissues, which ultimately affects drug distribution and disposition (Albermann et al. 2005).

Just like metabolizing enzymes, drug transporters are susceptible to genetic variability which contributes to the overall interindividual variation in the pharmacokinetics of therapeutic agents.

**Table 1-2 Main proteins involved in drug transport in humans.**

| Gene | Protein names | Tissue location | Substrates |
|---|---|---|---|
| ABCB1 | ABCB1, P-gp (P glycoprotein), MDR1 | Intestine, liver, kidney, brain, placenta, adrenal gland, testes | paclitaxel, diltiazem, digoxin, loperamide, ritonavir |
| ABCC2 | ABCC2, MRP2 | Intestine, liver, kidney, brain, placenta | etoposide, doxorubicin, ritonavir |
| ABCG2 | ABCG2, BCRP | Intestine, liver, breast, placenta | daunorubicin, methotrexate |
| SLCO1A2 | SLCO1A2, OATP1A2, OATPA | Brain | fexofenadin, ouabain |
| SLCO1B1 | SLCO1B1, OATP1B1, OATPC | Liver | benzylpenicillin, pravastatin, methotrexate, bilirubin, rifampicin |
| SLCO1B3 | SLCO1B3, OATP1B3, OATP8 | Liver | digoxin, ouabain, rifampicin |
| SLC22A1 | SLC22A1, OCT1 | Liver | acyclovir |
| SLC22A2 | SLC22A2, OCT2 | Kidney, brain | amantadine, serotonin |
| SLC22A4 | SLC22A4, OCTN1 | Liver, kidney, trachea | quinidine, verapamil |
| SLC22A5 | SLC22A5, OCTN2, SCD | Kidney, heart, placenta | l-carnitine, acetylcarnitine |
| SLC22A6 | SLC22A7, OAT1 | Kidney, brain | zidovudine, methotrexate |
| SLC22A8 | SLC22A8, OAT3 | Kidney, brain | ibuprofen, prostaglandin E2 |

### 1.1.3. Drug interactions

It is increasingly common to find individuals treated with not one but multiple therapeutic agents as certain aetiologies, such as hypertension, diabetes, HIV or transplantation, require treatments for life. Concomitantly administered drugs carry the risk of interacting with each other at many different levels and thereby impact on each other's pharmacokinetics. Drug-drug interactions (DDIs) may not be of clinical importance or may pose a serious health hazard.

An interaction can occur both between drugs themselves and between drugs and other compounds such as food and drink components or herbal remedies. The mechanism of the interaction can be direct, like competition for a transporter or displacement from a plasma protein; or indirect by modification of drug metabolizing enzymes expression or alteration of gastrointestinal pH or motility.

At the metabolic level, the most important type of interaction is the result of inhibition or induction of drug metabolizing enzymes.

Inhibitors can be competitive, when they bind to the enzyme active site displacing the original substrate; or mechanism based, when they form an irreversible covalent bond with the enzyme site preventing it from ever binding to their substrate again. There are some well-known drug inhibitors, like azole antifungals, macrolide antibiotics or HIV protease inhibitors (PIs). The most frequent outcome of an inhibition-type DDI is an increased exposure of the victim drug with higher potential for toxicity and accumulation.

The main risk of induction, on the other hand, is the reduction of plasma concentrations to non-therapeutic levels due to an enhanced metabolism of the drug. Induction requires the drug to interact with different cell components and start a signalling cascade that up-regulates transcription of the metabolic enzyme. This process requires time and therefore induction effects usually display a delayed presentation. Some of the most characterized enzyme inducers are rifampicin, carbamazepine, phenytoin and phenobarbital (Derwin et al. 2010).

It is especially important to monitor the effect of DDIs on drugs with a narrow therapeutic window and where plasma concentrations outside that window can pose a serious health risk, such as anticoagulants, antiarrhythmics, immunosuppressants or some HIV drugs. For these kinds of drugs therapeutic drug monitoring (TDM) may be advisable. When there is a good correlation between plasma concentrations and therapeutic effect or appearance of adverse events, TDM can guide dose adjustment and help management of DDIs. In some cases though, the co-administration of two drugs may be entirely contraindicated for safety or efficacy reasons.

Interestingly, DDIs can be beneficial in particular cases, such as the case of ritonavir, a CYP P450 inhibitor that is used to boost plasma concentrations of other HIV protease inhibitors (PI) and help them stay for longer in the therapeutic range thereby reducing the chance of selecting resistant strains of the virus (Wensing et al. 2010). Also, probenecid competes with penicillin for anionic transport in the kidney, which prolongs penicillin half-life (Robbins et al. 2012).

### 1.1.4. Pharmacogenetics

Genetics governs the expression of proteins, including drug metabolizing enzymes, transporters and plasma proteins. Moreover, physiological parameters such as blood flow, fat distribution or gastrointestinal motility may also be determined genetically. The combination of all these factors can result in significant differences in the bioavailability and clearance of drugs.

There is high inter-individual variability in the expression and activity of metabolic enzymes (Anzenbacher and Anzenbacherova 2001). In particular, cytochromes are highly polymorphic, their genotype can determine whether individuals are extensive or poor metabolizers, and in some cases, even ultra-rapid metabolizers. There is also variability in expression of transport proteins which determines distribution of drugs and their metabolites among body compartments.

Genetic variability at the molecular level can be of different types; single nucleotide polymorphisms (SNPs) consist of the substitution of one nucleotide for another in the genetic sequence; there are also insertions and deletions where a nucleotide is added or suppressed from the sequence. These small changes go mostly unnoticed because the majority of the genome consists of a non-coding sequence, but when one of those modifications happen on the promoter or coding regions of genes a phenotypic change may occur.

Copy number variation (CNV) is a form of structural variability that results in an abnormal number of copies of a genome region. Gene duplications result in a higher number of copies of a given gene and when the gene for a metabolic enzyme is affected the rate of metabolism of a particular drug may be increased (Redon et al. 2006).

The most frequent type of genetic variation are SNPs and their effect on protein expression and activity is very variable. When in the regulatory regions of the gene, they can affect expression of the protein by modifying affinity for transcription factors. When in an exon, or coding part of the gene, there are two options; they can be synonymous or non-synonymous. Non-synonymous mutations result in the change of an amino acid in the protein sequence, which may modify protein conformation or affinity for a substrate. Insertions and deletions can cause a shift of the reading frame of a gene which leads to the translation into a different amino acid sequence or perhaps an earlier or later stop codon may appear in the sequence. Genetic polymorphisms can also affect the splicing and stability of transcripts.

In humans, there are always two alleles of each gene, the paternal and maternal. The effect of a polymorphism will be different depending on how many copies an individual carries. The frequency of a SNP varies from population to population.

Pharmacogenetics tries to understand how genetic variability affects drug treatment (Evans and Relling 1999). However, it is difficult to determine the effect that one isolated polymorphism may have on a particular treatment, because many other factors intervene, including other genes and environmental effects.

Clinical interest for genetic testing needs to be assessed for every particular treatment. Dose optimization could be advised in cases where the presence of a polymorphism exerts a considerable effect on treatment efficacy or development of adverse events. There are a number of therapies where genetic testing has been recommended, for example CYP2C9 in warfarin treatment (Higashi et al. 2002).

The work presented in this thesis focuses on the pharmacogenetics of carboxylesterase 1 (CES1), a promiscuous esterase which is part of phase I liver metabolism. The genetic variability of this enzyme and its involvement in drug pharmacokinetics and pharmacodynamics are assessed and discussed.

## 1.2. Carboxylesterase 1

### 1.2.1. Protein classification

Carboxylesterase 1 (CES1) is involved in hydrolysing hydrophobic circulating compounds of either endogenous or exogenous origin into more polar molecules making them easier to eliminate from the system. CES1 belongs to the superfamily of α/β hydrolase enzymes widely present in living organisms. The numerous members of this superfamily vary in their structure and function but share an α/β fold secondary structure and a highly conserved catalytic triad of amino acids essential to fulfil their biological functions (Cygler et al. 1993).

Attempts to effectively identify and classify the numerous members belonging to the mammalian carboxylesterase family (EC 3.1.1.1) have been made. An initial classification assorted them in A, B and C esterases, based on their substrate specificity (Aldridge 1953). However, overlapping was frequent and a new and more precise classification based on sequence homology of the coding gene was proposed. Mammalian carboxylesterases are currently divided into four main groups and several subgroups (Satoh and Hosokawa 1998).

Function, gene regulation, tissue distribution and subcellular location are very varied within this superfamily of enzymes. Some members are very selective about their substrates, others very promiscuous; some are expressed in multiple cell types, others have precise expression locations; some are attached to intracellular organelles, others are synthesized to be excreted into plasma (Satoh et al. 2002)

In human metabolism, CES1 plays an important role. It is the main hydrolysing enzyme present in the liver as well as having a variety of other functions. These functions are represented by the number of different names by which CES1 enzyme is known: cholesteryl ester hydrolase (CEH), retinyl ester hydrolase (REH), triacylglycerol hydrolase (TGH), acyl-coenzymeA:cholesterol acyltransferase (ACAT), human monocyte/macrophage serine esterase (HMSE), methylumbelliferyl-acetate deacetylase 1, egasyn or cocaine carboxylesterase.

A closely related enzyme, carboxylesterase 2 (CES2), also plays an important role in human metabolism; its overall expression, tissue location and substrate specificity generally differs but can in some cases overlap that of CES1 (Satoh and Hosokawa 1998).

A summary of the characteristics of the main human carboxylesterases can be found on Table 1-3.

**Table 1-3 Main human carboxylesterases**

| Name | Symbol | Main location | Substrate specificity | Specific substrate |
|---|---|---|---|---|
| Carboxylesterase 1 | CES1 | Liver | Bulky acyl group<br>Small alcohol group | Methylphenidate |
| Carboxylesterase 2 | CES2 | Small intestine | Small acyl group<br>Bulky alcohol group | Prasugrel |
| Carboxylesterase 3 | CES3 | Brain | N/A | N/A |

### 1.2.2. Tissue expression

The distribution of carboxylesterases in the human body is ubiquitous, although the expression of the particular members of the family is tissue specific, and can determine their role in metabolism (Satoh and Hosokawa 1998).

CES-1 is mainly expressed in the liver and is quantitatively the major human hydrolysing enzyme; it is also present on a smaller scale in macrophages and lungs (Imai et al. 2003, Crow et al. 2008); CES-2, on the other hand, is expressed mainly in small intestine, but also in kidney, heart, skeletal muscle and liver (Zhang et al. 2002). There is a third carboxylesterase, CES3, that has been found primarily in brain tissue and it may play an important role in the blood brain barrier, preventing highly lipophilic compounds from entering the brain (Mori et al. 1999).

Although esterase activity has been found in human plasma, it is due to other members of the α/β hydrolase superfamily, such as butyrylcholinesterase or paraoxonase. CES1 is not found circulating in human plasma but is instead mostly found attached to endoplasmic reticulum (ER) (Li et al. 2005). The CES1 C-terminal region, HXEL-COOH (His-Xxx-Glu-Leu-COOH), interacts with a receptor found in ER lumen, EDR receptor (Endoplasmic reticulum retention receptor). The HXEL domain is analogous to that of other proteins that are retained in ER. In carboxylesterases when that terminal fragment is hydrolysed or absent as a result of a mutation the protein is secreted (Satoh and Hosokawa 1998). In addition, CES1 has also been found in cytosol (Satoh et al. 2002).

### 1.2.3. Physiological roles

The main physiological role of CES1 is the metabolism of xenobiotics. CES1 plays an important role in the first pass effect of multiple drugs due to its remarkable hydrolysing capacity. It quickly hydrolyses ester, amide, thioester and carbamate bonds to their more hydrophilic acids.

The effects of this enzyme are also considered protective as it clears human plasma of environmental toxicants and carcinogens. Presumably, that is the reason why this group of enzymes has been evolutionary successful in so many species (Hosokawa et al. 2007).

Multiple therapeutic compounds are cleaved by CES1. The enzyme can be part of the overall metabolic pathway leading to drug elimination or it can be involved in the activation of compounds that have been formulated as ester prodrugs to increase their lipophilicity and improve absorption (Satoh and Hosokawa 1998).

Furthermore, CES1 plays an important role in lipid metabolism thanks to its cholesterol ester hydrolase activity (CEH) which modulates the equilibrium between cholesterol esters and free cholesterol in macrophages and liver (Ghosh 2000). Other endogenous compounds that get hydrolysed by CES1 are short and long-chain acyl-glycerols, long-chain acylcarnitine and long-chain acyl-coA esters (Satoh and Hosokawa 1998).

In addition to its hydrolysing capacity, CES1 is also capable of carrying out transesterification reactions. Its acyl-coA:acyl transferase (ACAT) activity holds high physiological importance for fatty acid metabolism (Becker et al. 1994). This ability can, unfortunately, be a source of toxicity if it occurs in the presence of ethanol.

CES1 is also involved in trafficking and retention of proteins in ER; for instance, C-reactive protein (CRP) is retained in the ER luminal side by an interaction with CES1. The affinity between these two proteins decreases during the acute inflammatory phase response and this results in the secretion and consequent increase of CRP in plasma (Macintyre et al. 1994). CES1 also forms a complex with β-glucuronidase, a phase II metabolism enzyme. This interaction is believed to enhance the processing of compounds that are a target for both these enzymes (Novak et al. 1991).

Apart from the physiological roles described above, a number of potential therapeutic uses of this enzyme have been proposed. CES-1 could be exploited as a drug target to modulate the activity of therapeutic agents metabolized by it or used directly as a drug, e.g. in the treatment of cocaine overdose, as it is the main deactivator of this agent *in vivo*. Also, it can be administered as prophylaxis against chemical weapons when organophosphates such as cyclosarin or soman are used; these compounds inhibit acetyl cholinesterase irreversibly producing death by cardio-respiratory failure, and pre-administration of CES1 could confer a protective effect by degrading these warfare chemicals (Hemmert et al. 2010).

### 1.2.4. Protein structure

CES1 protein bears high structural resemblance to other human and animal esterases. The most highly conserved region corresponds to the active site of the enzyme, a catalytic triad composed by the amino acids serine, histidine and glutamic acid.

The elucidation of the crystal structures of the first mammalian carboxylesterase: rabbit CES1, (Bencharit et al. 2002) and human CES1 (Bencharit et al. 2003) opened the door for a better understanding of the structure, regulation and mechanism of action of this enzyme.

The protein is composed of three domains: a catalytic domain, where the catalytic pocket and the characteristic α/β fold sheets are located; a regulatory domain and a α/β domain (Bencharit et al. 2003). Each monomer (64 kD) of the enzyme contains all three domains. The active enzyme associates in trimers that exist in equilibrium with an hexameric form (Bencharit et al. 2003).

In human CES1 the three amino acids that carry out the two-step reaction at the catalytic pocket are Ser221, His468 and Glu354. The catalytic pocket is a hydrophobic cavity formed in the interface of the three domains. Among the amino acids that form this pocket we find what is called the oxyanion hole, a group of three glycines (Gly141, Gly142, Gly143) whose role is key to stabilizing the reaction intermediates. In fact, a naturally occurring polymorphism where the glycine in position 143 is substituted by a glutamic acid results in a low activity variant of the protein (Zhu et al. 2008). The genetic variability of CES1 enzyme will be discussed in more detail in a later section.

The two major cavities of the catalytic pocket are a large flexible groove and a smaller rigid one. These two cavities accommodate the acyl and alcohol group of each compound and determine substrate specificity. The ‘side door’ is an opening at the catalytic pocket which is different from the entrance and is thought to help accommodate big substrates, as well as allowing quick entrance and release of reagents and products to achieve a high processing speed.

Additionally, there is a second ligand binding site different from the catalytic pocket known as the Z site on the surface of the protein. It consists of a low affinity ligand binding region that self-associates to form the hexamer conformation; this equilibrium is displaced towards the trimeric form in the presence of molecules of ligand.

Other structural features worth mentioning are the ER lumen-binding sequence (KXEL) explained previously in the tissue distribution section, and the glycosylation site; an N-linked high type glycosylation position that binds a single mannose residue on Asn79. It is believed that this is essential for enzyme activity (Kroetz et al. 1993).

### 1.2.5. Enzymatic mechanism

Ser221, His468 and Glu354 participate in the two-step hydrolytic reaction where a molecule containing an ester bond is cleaved into an alcohol and a carboxylic acid.

The molecular mechanism takes place thanks to an increase in the nucleophilicity of the oxygen in the serine residue that attacks the carbonyl group of the ester bond which relocates its charge forming a tetrahedral intermediate.

The negatively charged oxygen lowers the charge forming a double bond, and forces the release of the alcohol product. The acyl part of the molecule stays covalently linked to the serine. It will be later attacked by a nucleophile, usually water, forming a second tetrahedral intermediate that will relocate its charge as previously, this time, releasing the carboxylic product and reverting the amino acids in the catalytic pocket to their original state (Satoh and Hosokawa 1998).

It is possible for the second nucleophile to be a molecule other than water, for example, ethanol; the products released from such a transesterification reaction may be highly toxic compounds like cocaethylene which results in the event of cocaine and ethanol co-consumption (Brzezinski et al. 1994).

### 1.2.6. Substrate specificity

The members of the α/β hydrolase superfamily are generally characterized by considerable promiscuity and overlap in terms of substrate specificity. Nevertheless, there are members that show high selectivity towards particular substrates like acetyl cholinesterase or juvenile hormone esterase.

In the same way, even though some substrates are susceptible to hydrolysis by any esterase, there is usually one enzyme that predominates in their metabolism and represents the major hydrolytic pathway for that compound (Satoh et al. 2002).

The preference of each enzyme for a particular substrate is generally determined by the structure on either side of the ester bond. If the group attached to the carbonyl functional group, also known as acyl group, is large, that compound will be metabolized by CES1 rather than CES2 and vice versa.

Most compounds will be susceptible to be processed by both enzymes although there are some examples like methylphenidate or prasugrel that are preferentially targeted by CES1 and CES2, respectively (Srinivas et al. 1992, Kazui et al. 2010).

### 1.2.7. Therapeutic compounds

The range of molecules susceptible to be metabolized by CES1 is varied in structure and indication. There are anaesthetics like lidocaine (Alexson et al. 2002, Fujiyama et al. 2010), immunosuppressants like mycophenolate mofetil (Fujiyama et al. 2010), psychostimulants like methylphenidate (Srinivas et al. 1992, Fujiyama et al. 2010), drugs of abuse like cocaine (Kamendulis et al. 1996) and so on. It is remarkable though how many compounds used to treat cardiovascular conditions are substrates for this enzyme, for example, angiotensin-converting-enzyme inhibitors (ACEIs) like imidapril and delapril (Takai et al. 1997); angiotensin receptor blockers (ARB), like cardesartan and olmesartan (Nishikawa et al. 1997, Ma et al. 2005); fibrates like clofibrate, fenofibrate; statins, like lovastatin, simvastatin (Vickers et al. 1990, Halpin et al. 1993); or anticoagulant agents like clopidogrel and dabigatran (Tang et al. 2006, Blech et al. 2008).

Most therapeutic compounds hydrolysed by CES1, contain ester bonds that are cleaved by the enzyme, but there are some with amide linkages, like the anticonvulsant rufinamide (Williams et al. 2011), and with carbamate linkages such as the antitumorals capecitabine (Tabata et al. 2004) and irinotecan (Satoh et al. 1994).

Some CES1 substrates are inactivated by the action of CES1 like the GABA antagonist flumazenil (Kleingeist et al. 1998) or the opiate meperidine (Zhang et al. 1999); others require hydrolysis of their ester/amide moiety to become active like the immunosuppressant ciclesonide (Mutch et al. 2007) or the antiviral oseltamivir (Shi et al. 2006).

Although already numerous, the list of CES1 substrates is likely to continue growing as the strategy of including ester bonds to create prodrugs with enhanced lipophilicity is still used when formulating new therapeutic agents.

### 1.2.8. CES1 gene

In a similar way that esterase protein structure shows remarkable homology among members of the same family, the genes that encode for these enzymes also show rather high sequence identity (Satoh and Hosokawa 2006), for instance, acetyl cholinesterase, CES2 and CES3 show 37%, 48% and 77% sequence identity with CES1, respectively.

In humans, CES1 is encoded by the CES1 gene located on chromosome 16, which consists of three isoforms: CES1A1, CES1A2, CES1A3 (Hosokawa et al. 2007, Yoshimura et al. 2008). It is believed that the three isoforms originally come from one. They originated by a phenomenon of gene duplication and evolved independently after that. It has been suggested that CES1A2 is a duplication of CES1A3 gene rather than CES1A1 (Fukami et al. 2008).

The CES1A1 and CES1A2 genes (AB119997 and AB119998) are inversely located on chromosome 16, they are both functional and encode for almost identical transcripts, the mature protein is the same (Tanimoto et al. 2007). CES1A3 is a pseudogene with a premature stop codon in exon 6. Consequently, both CES1A1 and CES1A2 genes contain 14 exons and span 30 kbp (kilobase pairs), while CES1A3 only has 6 exons and spans 14 kbp. The genetic sequence following the stop codon of CES1A3 is highly similar to exons 7 to 14 of CES1A1 and CES1A2 isoforms.

The difference between CES1A1 and CES1A2 resides mainly in exon 1 and the promoter region which determines the fact that although the final product of CES1A1 and CES1A2 is an identical mature protein of 567 amino acids, the majority of CES1 protein present in the liver is a product of CES1A1. CES1A2 transcription efficiency is 2% of that of CES1A1, therefore, only genetic variants present on the CES1A1 genomic region are likely to produce a significant impact on CES1 activity (Hosokawa et al. 2008, Yoshimura et al. 2008).

### 1.2.9. Genetic variability

Synthetic recombinant CES1 enzymes have been developed where one or more of the three amino acids that compose the catalytic triad (Ser221, Glu 354, and His 468) were mutated. These displayed markedly impaired catalytic activity which proves the importance of those amino acids in the hydrolytic reaction (Satoh and Hosokawa 2006). In addition, the effect of naturally occurring polymorphisms on enzyme activity has been explored and reported in the literature.

Geshi et al. found that the -816A>C polymorphism present in the promoter region of CES1 was significantly associated with the efficacy of imidapril (Geshi et al. 2005). Later Yoshimura et al. speculated that this polymorphism could be a marker for other functional polymorphisms present in promoter regions with sp1 transcription binding sites, where the real functional SNPs would be located (Yoshimura et al. 2008). The same SNP was later evaluated for its potential effect on irinotecan therapy, but no effect was found. The reason could be that the polymorphism was present on the CES1A3 pseudogene region, the impact of a mutation is likely to be different depending on what isoform it is located (Fukami et al. 2008).

Zhu et al. found two genetic variants when they were exploring the effect of ethanol on methylphenidate pharmacokinetics. One of them Arg260fs was a frame shift mutation that resulted in a truncated protein with no activity whatsoever. The frequency of this polymorphism was extremely low. The second one, Gly143Glu, resulted in a low activity variant. The glycine in position 143 was substituted by a glutamic acid. This glycine is part of the oxyanion hole and therefore participates in the stabilization of reaction intermediates, its absence therefore affects the hydrolysing capacity of the enzyme (Zhu et al. 2008). This non-synonymous polymorphism has been explored in a number of other cohorts, and its effects characterized in treatments with several other therapeutic agents, where it consistently appears to have a deleterious effect on the catalytic efficiency of the enzyme (Zhu and Markowitz 2009, Walter Soria et al. 2010, Tarkiainen et al. 2012, Lewis et al. 2013).

In 2010 Yamada et al. found a relationship between SNP rs3815583 and isoniazid-related hepatotoxicity (Yamada et al. 2010). This SNP located in -75 T>G in the 5' untranslated region (5'-UTR) region of CES1A1 was later linked to irinotecan treatment (Sai et al. 2010) and to appetite reduction in children treated with methylphenidate (Bruxel et al. 2012).

Recently, Pare et al. found a significant relationship between SNP rs2244613 and the pharmacokinetics of dabigatran (Pare et al. 2013).

Even though there are increasing numbers of reports exploring the effect of genetic variability on the treatment with different drugs metabolized by CES1 it is not comparable to the amount of evidence generated for other liver metabolizing enzymes, particularly CYP P450 enzymes. Moreover, there are more than 900 CES1 genetic variants in the dbSNP database but their functional implications have not been systematically evaluated yet.

### 1.2.10. Physio-pathological conditions

There are other factors that contribute to CES1 variability in activity and expression. For instance, some studies show that the expression of CES1 and CES2 varies throughout the developmental process with new-borns displaying lower expression and higher inter-individual variability of CES1 and CES2 than children (1-10 years old) or adults (>18 years old) (Yang et al. 2009). Some authors have also suggested a differential expression of carboxylesterases between sexes, where women appear to show higher catalytic efficiency than men (Patrick et al. 2007) .

Furthermore, differences in the ability to hydrolyse carboxylesterase substrates have been reported in the process of pathological conditions affecting the liver. It has been shown for example that the CES1-mediated activation of perindopril and cilazapril was reduced in patients suffering from hepatitis and cirrhosis (Thiollet et al. 1992, Gross et al. 1993, Prandota 2005). Interestingly, the expression of CES1 has been shown to be increased in the case of infection with hepatitis C virus, where the assembly phase of the virus is highly dependent on the formation of lipid droplets in which CES1 may play an important role, thus an increased expression of the enzyme appears to be beneficial for viral replication (Blais et al. 2010).

### 1.2.11. Regulation

Expression of enzymes and transporters involved in human metabolism is often regulated endogenously via nuclear receptors such as PXR (pregnane X receptor), CAR (constitutively activated receptor) or GR (glucocorticoid receptor). They bind a wide range of ligands that enter the body and then transpose into the cell nucleus where they interact with DNA response elements to upregulate and downregulate genes that encode for enzymes and transporters involved in xenobiotic metabolism (Handschin and Meyer 2003).

There could also be endogenous regulation of carboxylesterases. Hormones like testosterone, oestrogen, insulin, glucagon or pituitary hormones have been shown to have an effect on carboxylesterase expression in mice (Satoh and Hosokawa 1998). These hormonal mechanisms in humans have not been extensively explored to date.

Some cytokines like TNF-α (tumour necrosis factor alpha) and IL6 (interleukin 6) that are released during inflammatory processes have been shown to modify the expression of enzymes, transporters and nuclear receptors themselves, affecting metabolic reactions. A transcriptional suppression of the CES1 gene has been reported in primary hepatocytes and HepG2 cells in the presence of IL-6 (Yang et al. 2007).

The presence of the bacterial endotoxin lipopolysaccharide (LPS), which induces the expression of the pro-inflammatory cytokines (TNF-α, IL6) in the liver, has also been shown to decrease CES1 and CES2 mRNA and protein levels in a time dependent manner. (Mao et al. 2011).

### 1.2.12. DDIs and CES1

The effect of some therapeutic agents on CES1 expression has been assessed. Dexamethasone and rifampicin appear to moderately induce CES1 and CES2 expression in cultured human hepatocytes (Zhu et al. 2000).

Furthermore, there are several reports about inhibitors of CES1, including some of the actual substrates of the enzyme (Shi et al. 2006, Tang et al. 2006, Fukami et al. 2010, Zhu et al. 2010, Hatfield and Potter 2011, Rhoades et al. 2012). There also appears to be some inhibition of CES1 with grapefruit juice (Li et al. 2007, Fukami et al. 2010).

Finally, it is worth mentioning the potentially dangerous interaction between enzyme substrates and ethanol, which is the most studied interaction to date. Co-administration of any CES1 substrate and ethanol has the potential of releasing into plasma products of the transesterification rather than the hydrolytic reaction. It is well characterized that this is an interaction between cocaine and ethanol with the release of the highly toxic cocaethylene (Bencharit et al. 2006).

The transesterification of fatty acyl CoA molecules in the presence of ethanol, which can happen due to a chronic alcohol abuse, releases fatty acyl ethyl esters (FAEE) and may cause necrotic liver decay (Lange and Sobel 1983).

Methylphenidate activation into its active metabolite (ritalinic acid) is performed via CES1 in a stereospecific manner. Co-administration of methylphenidate and ethanol can modify the balance of enantiomers present in plasma as well as cause the release of the metabolite ethylphenidate. Ethanol could also inhibit the reaction resulting in higher plasma concentrations of the parent compound (Patrick et al. 2007).

### 1.3. Aims of the thesis

Although a major player in the metabolic processing of numerous endogenous compounds and xenobiotics, carboxylesterases remain largely unexplored. More evidence is necessary to functionally and clinically characterize the factors that affect CES1 metabolic performance and its repercussions for drug response.

The aim of this thesis is to improve our understanding of some of the factors that contribute to the variability of the expression and hydrolytic capacity of CES1 enzyme, focusing especially on the role of genetic variability and DDIs on enzyme expression, catalytic efficiency, drug pharmacokinetics and pharmacodynamics.

The contents of the thesis are distributed a follows:

- Chapter 2 : Development and validation of methodology
- Chapter 3: Assessment of the impact of genetic variants of CES1 gene on the pharmacokinetics and pharmacodynamics of treatment with the antiplatelet agent clopidogrel.
- Chapter 4: Exploration of the possibility of a DDI between clopidogrel and NNRTIs mediated by CES1.
- Chapter 5: Assessment of the impact of genetic variants and HIV/TB infected status on isoniazid pharmacokinetics.
- Chapter 6: Investigation of the effect of rifampicin, rifabutin and rifapentine on CES1 and CES2 mRNA expression.
- Chapter 7: Final discussion of results

# CHAPTER 2

# Methods

# Chapter 2: Methods

## 2.1. Introduction

Accurate measure of biochemical parameters requires the utilization of reliable and robust techniques, appropriately optimized and validated to generate sound measures of biochemical and genetic determinants that along with demographic, phenotypic and clinical data will assist us to further understand the underlying reasons of variability in treatment response as well as allow us to explore the mechanisms of drug-drug interactions (DDIs).

In this chapter is detailed, firstly, the optimization and validation of a High Performance Liquid Chromatography technique with ultraviolet detection (HPLC/UV) for the characterization and quantification of clopidogrel main circulating metabolite: clopidogrel carboxylic acid (CLPM). The technique will be used in the thesis to elucidate the impact of carboxylesterase 1 (CES1) genetic variability (Chapter 3) and antiretroviral co-medications (Chapter 4) on clopidogrel pharmacokinetics.

Secondly, in this chapter is described the process of optimization and validation of a real-time polymerase chain reaction (qPCR) allelic discrimination assay for the genotyping of rs71647871 and rs71647872 genetic variants. SNP genotyping will be a core technique to this thesis work as we explore the effect of CES1 pharmacogenetics in the treatment with clopidogrel (Chapter 3) and of tuberculosis (Chapter 5).

## 2.2. HPLC-UV method for the quantification of clopidogrel main circulating metabolite in human plasma

### 2.2.1. Introduction

Clopidogrel (CLP) is indicated for the prevention of cardiovascular complications in individuals after suffering acute coronary syndrome (ACS) and to avoid stent thrombosis after undergoing percutaneous coronary intervention (PCI).

CLP is a second generation thienopyridine, a family of therapeutic agents that act by irreversibly inhibiting the $P_2Y_{12}$ adenosine diphosphate (ADP) receptor expressed on platelet surface, thus interfering with the coagulation cascade.

CLP bisulphate is orally administered and quickly absorbed and metabolized. A two-step enzymatic transformation carried out by hepatic cytochromes (CYP P450), such as CYP2C19 and CYP3A4, is required to yield the pharmacologically active metabolite (Kazui et al. 2010). Nevertheless, the vast majority of parent compound undergoes hydrolysis by hepatic carboxylesterase 1 (CES1) which turns about 85% of all circulating CLP into the inactive carboxylic derivative (CLPM) (Tang et al. 2006).

Efforts have been made to detect both the parent compound and active metabolite (R-130964). However, the former disappears rapidly from plasma following absorption, and the latter is highly labile and plasma concentrations are low. Such concentrations have only been detected with highly sensitive detection methods such as liquid chromatography-mass spectrometry tandem technique (LC/MS-MS) (Nirogi et al. 2006, Robinson et al. 2007, Takahashi et al. 2008, Peer et al. 2012).

Therefore the indirect approach of determining CLPM, the inactive and more abundant metabolite product of CES1 has been used to study CLP pharmacokinetics and can serve as a means to explore CES1 activity. The possibility of using CLPM concentrations as a tool to separate non-compliance from other factors responsible for the considerable interindividual variability in CLP treatment has also been suggested (Mani et al. 2008).

Previously published methods have quantified CLPM using HPLC/UV detection in rat plasma (Singh et al. 2005), in human serum (Bahrami et al. 2008) and in human plasma for pharmacokinetic studies in healthy volunteers (Souri et al. 2006, Rouini et al. 2009).

The purpose of the work described in this section was to develop and validate a reverse phase HPLC/UV detection method for the quantification of CLPM in a low human plasma volume as a tool to first, improve our knowledge of CES1 pharmacogenetics (PGx) involvement in CLP pharmacokinetic/pharmacodynamic (PK/PD) relationship (Chapter 3); and second, explore the possibility of previously unrecognized interactions between CLP and non-nucleoside reverse transcriptase inhibitors (NNRTIs), used in the treatment of human immunodeficiency virus (HIV) (Chapter 4).

### 2.2.2. Materials and methods

#### 2.2.2.1. Instrumentation

The HPLC system used for the determination and quantification of CLPM consisted of an HPLC pump system P680, an autosampler ASI-100 and a UV detector UVD 170U, all from Dionex (Sunnyvale, California, USA). The software Chromeleon v6.8 was used for management of the HPLC system. Separation was achieved using a Hypersyl gold 150x4.6 mm column, with a particle size of 5 µm obtained from Thermo Scientific Inc. (Waltham, Massachusetts, USA).

#### 2.2.2.2. Chemicals

CLPM powder was obtained from Toronto Research Chemicals Inc. (Toronto, Canada). Drug free blank plasma was obtained from healthy donors from the Liverpool blood bank (Liverpool, UK).

All other chemicals used for stock solution preparation, extraction and mobile phase composition were of analytical grade. Methanol, ethyl acetate and acetonitrile (ACN) were purchased from Sigma-Aldrich (St. Louis, Missouri, USA); orthophosphoric acid (OFA) 85% HiPerSolv CHROMANORM® and formic acid 99-100% AnalaR NORMAPUR® were obtained from VWR (Radnor, Pennsylvania, USA).Water was distilled by Purelab option Q system (Elga labwater, High Wycombe, UK).

### 2.2.2.3. Stock solutions, Standards and Quality Controls

CLPM powder, with a purity of 98%, was kept at -20°C and protected from humidity due to its hygroscopic properties.

The appropriate amount of powder was dissolved in 100% methanol to produce a stock solution with a final concentration of 1 mg/ml. The stock solution was stored at -20°C.

Quality control (QC) solutions (6000, 800 and 300 ng/ml) and a calibration standard (10,000 ng/ml) were prepared in plasma and stored at -20°C until use, within 1 month. The stock standard solution was subsequently diluted in the same matrix to obtain the calibration curve in the range of 78 to 10,000 ng/ml.

### 2.2.2.4. Standards, Quality controls and sample preparation

For every standard, QC and study sample, 200 µl were aliquoted into 7 ml round bottom screw top glass tubes. 2 ml of ethyl acetate and 50 µl of formic acid were added to each tube. They were tumbled for 30 minutes and subsequently spun down for 5 minutes at 4,000 rpm. Supernatant was decanted into clean open glass tubes and samples were dried in a vacuum centrifuge at a temperature of up to 55°C.

The residue was later resuspended in 200 µl of reconstitution solution, a mixture of ACN and $H_2O$ (25:75, v/v), and transferred into analysis vials. Finally, vials were spun down at 4,000 rpm for 5 minutes. 100 µl were injected into the chromatographic system.

### 2.2.2.5. Chromatographic conditions

A gradient method was applied with varying concentrations of aqueous ($KH_2PO_4$ 10 mM with 200 µl OFA, pH=3.2) and organic (ACN: $H_2O$, 90:10) solvents. Details of the gradient can be found in Table 2-1. Both solvents were prepared fresh every day. The aqueous buffer was elaborated after dilution and acidification of a $KH_2PO_4$ 1M stock solution, which had been prepared in aseptic conditions and kept at 4°C.

The chromatographic separation took place at a flow rate of 1 ml/min and lasted a total of 9.2 minutes. The detection wavelength was 200 nm. All separations were performed at room temperature.

**Table 2-1 Chromatographic separation solvent concentration-time gradient**

| **Time (min)** | **Aqueous solvent (%)** ($KH_2PO_4$ 10 mM + OFA (pH =3.2)) | **Organic solvent (%)** (ACN 90% $H_2O$ 10%) |
|---|---|---|
| 0 | 85 | 15 |
| 0.5 | 74 | 26 |
| 4 | 73 | 27 |
| 4.1 | 15 | 85 |
| 7.1 | 15 | 85 |
| 7.2 | 85 | 15 |
| 9.2 | 85 | 15 |

### 2.2.2.6. Calibration curve

The calibration curve was obtained plotting the height of the CLPM peaks after extraction and chromatographic separation of 8 concentration points within the range of 78 to 10,000 ng/ml.

Standard curve equations were derived using the best fitted equation model for least-squares regression analysis.

Assay variation for low, medium and high QC samples (LQC, MQC and HQC) was expressed in terms of coefficient of variation [CV% = (standard deviation/mean) × 100]. As per U.S. Food and drug administration (FDA) regulations mean target concentrations of all calibration points should be within ±15% (±20% for LOQ) of the nominal value and %CV should not exceed 15% (20% for LOQ) (FDA 2001).

### 2.2.2.7. Bioanalytical method validation

To test specificity and selectivity of the method, drug-free plasma samples from four different sources were processed and injected into the HPLC system to assess the degree of interference between the analyte and any endogenous plasma component.

Accuracy and precision were determined with six sets of QCs per session (LQC, MQC, and HQC). Accuracy was expressed as the percent variation from nominal concentration. Precision was expressed as the coefficient of variation (CV %) at each QC concentration.

Extraction efficiency of CLPM from plasma was determined by comparing the peak height of the drug extracted from the three QCs, with those obtained by direct injection of the same amount of drug in the reconstitution solution with the same composition as the mobile phase. Four batches with 6 aliquots at each of the three QC levels were assayed, a total of 24 samples per QC level. According to FDA guidelines recovery needs not be 100% but it should be consistent, precise and reproducible. It was allowed a variation between 85% and 115%.

Stability studies were performed for the stock solution comparing the old stock and a freshly prepared stock at a concentration of 5,000 ng/ml.

To assess freeze/thaw stability, 6 aliquots at the three QC levels (LQC, MQC, HQC) after 3 freeze/thaw cycles were compared to freshly prepared ones. In each freeze/thaw cycle samples were stored at -20ºC and then thawed at room temperature, when completely thawed, samples were re-frozen for 12-24 hours at -20ºC.

To assess bench top stability, 6 aliquots at the three QC levels (LQC, MQC, and HQC) kept on the bench for 24 hours were analysed against a freshly prepared calibration curve.

For processed sample stability evaluation 6 aliquots of QC samples were processed and kept in the fridge at 4ºC for 24 hours and analysed against a freshly prepared standard curve.

To assess re-injection reproducibility, samples were kept in the autosampler at room temperature for 24 hours and analysed against a freshly prepared calibration curve.

Previously published data was relied upon for long term stability of CLPM and was not evaluated in this study. The analyte had been reported to be stable for at least 1.5 years (Lagorce et al. 1998).

### 2.2.3. Results

As shown in Figure 2-1 the total chromatographic run time was 9.2 minutes and CLPM retention time was 5.4 minutes.

A linear least-squares regression analysis was performed and the mean regression coefficient ($r^2$) was 0.9993. The assay LOQ and LOD were 156 ng/ml and 78 ng/ml, respectively. The response was linear up to 10,000 ng/ml. Calibration standard curve and QCs are presented in Figure 2-3.

No significant interfering peaks were observed at the analyte retention time in the four different drug-free plasma samples tested. Figure 2-2 shows a zoom on the four drug-free plasma aliquots and a low concentration standard (312 ng/ml) of CLPM at retention time (5.4 minutes).

The results for accuracy and precision can be found in Table 2-2. All observed data were below 15% variation, in accordance with FDA guidelines.

Mean extraction efficiencies of CLPM from plasma at HQC, MQC and LQC levels after testing four batches of six aliquots each were 92%, 92% and 107% respectively, with an overall extraction efficiency of 97% and a mean %CV of 9.16% showing that analyte recovery was reproducible across batches.

Comparison of old stock solution with freshly prepared stock at 5,000 ng/ml showed a variation in concentration of less than 1%.

Freeze-thaw stability was confirmed after observing that the mean difference in peak height between 6 aliquots at each of the QC levels that had been subjected to 3

freeze-thaw cycles and freshly prepared ones was below 15%, the mean difference in peak height was 7.7%, 14.9% and 12.3% for LQC, MQC and HQC respectively.

Bench top stability was confirmed after observing that the variation in concentration between samples at the 3 QC levels kept 24 hours on the bench and analysed against a freshly prepared calibration curve did not exceed 15%, the mean difference in concentration was 14.8%, 9.6% and 9.8% for LQC, MQC and HQC respectively.

Processed sample stability was confirmed after observing that the difference in concentration between 6 processed aliquots at each of the 3 QC levels kept 24 hours at 4ºC and freshly prepared ones did not exceed 15%, it was 3.1%, 12.4% and 6.2% for LQC, MQC and HQC respectively.

Re-injection reproducibility was confirmed after observing that the difference in mean concentration values between 6 processed aliquots at the 3 QC levels kept in the autosampler for 24 hours and freshly prepared ones did not exceed 15%, it was 2.8%, 5.1% and 3.0% for LQC, MQC and HQC respectively.

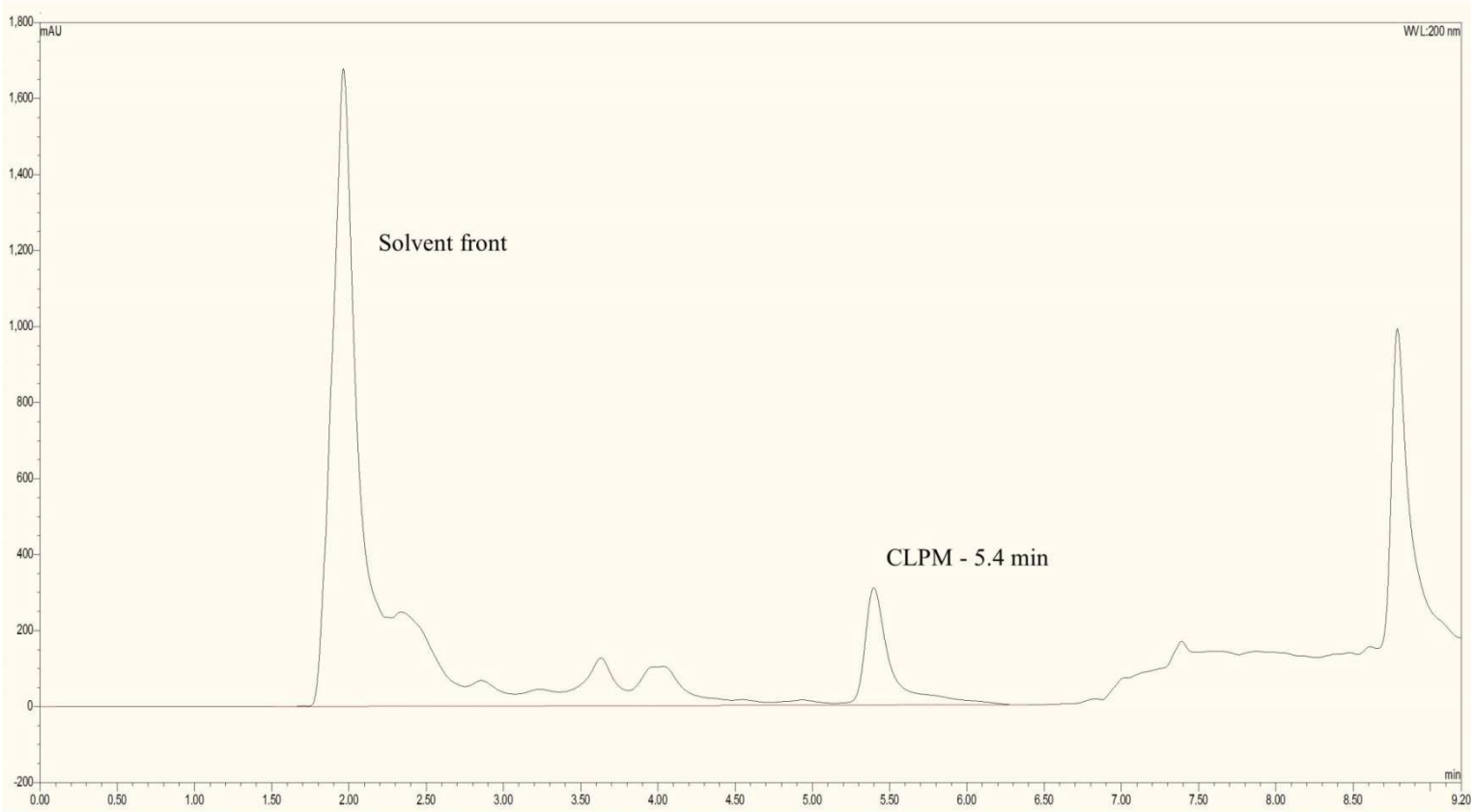


**Figure 2-1 HPLC chromatogram of CLPM metabolite.** x axis is time in minutes, y axis is UV absorbance in mV. The first peak at 2 minutes is the solvent front. CLPM retention time is 5.4 minutes. Total run time is 9.2 minutes.

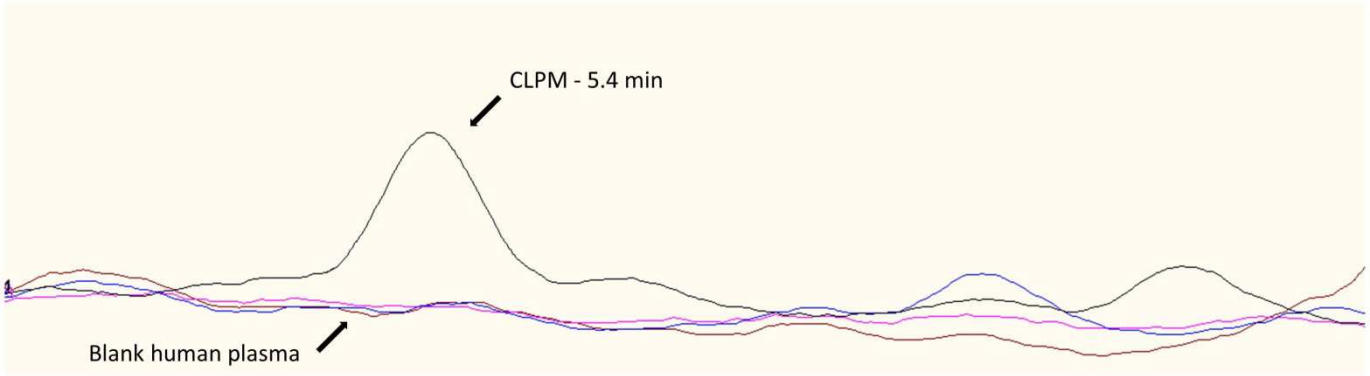


**Figure 2-2 Chromatographic detail of 5 plasma samples:** drug-free human plasma from 4 donors and plasma sample spiked with CLPM metabolite at a concentration of 312 ng/ml. x axis is time in minutes, y axis is UV absorbance in mV.

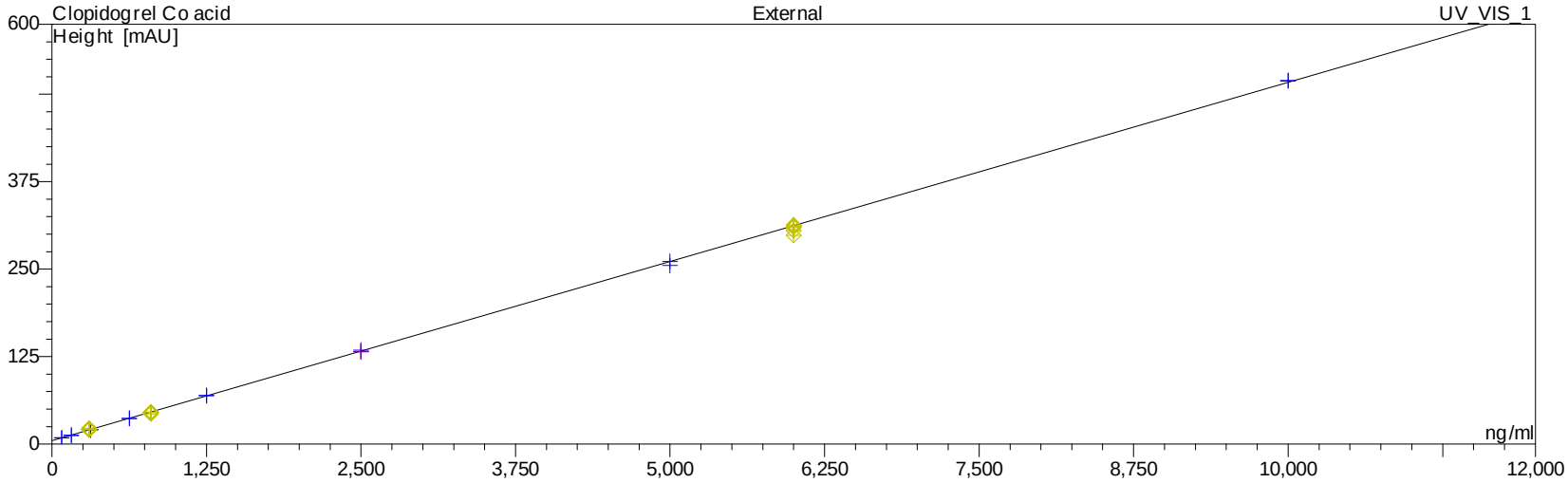


**Figure 2-3 Standard curve and QCs of the HPLC/UV method for CLPM detection.** Linearity of the method was confirmed between 78 ng/ml and 10,000 ng/ml. Mean regression coefficient ($r^2$) was 0.9993. LQC, MQC and HQC were 300 ng/ml, 800 ng/ml and 6,000 ng/ml, respectively. x axis is concentration in ng/ml, y axis is UV absorbance in mV.

**Table 2-2 Validation results of HPLC/UV assay: accuracy, interday and intraday precision**

| Analyte | QC | Accuracy (%) | Precision (CV %) | |
|---|---|---|---|---|
| | | | Interday | Intraday |
| CLPM | LQC | -1.04 | 8.27 | 8.42 |
| | MQC | -3.99 | 9.26 | 4.35 |
| | HQC | 1.49 | 5.93 | 2.32 |

### 2.2.4. Discussion

Several methods for the quantification of CLPM using HPLC with UV detection have been published. Singh et al. reported a method to quantitate CLPM in Wistar rat plasma. The method involved a liquid-liquid extraction, was sensitive (125 ng/ml) and with small sample volume requirements, however the total run time was 20 minutes which limits its utility for studies with large numbers of samples (Singh et al. 2005).

Souri et al. published a method with a considerably shorter run time, 11 minutes, slightly less sensitive (200 ng/ml) and with large sample volume requirements (500 µl) (Souri et al. 2006).

Bahrami et al. managed to reduce the total run time to 5.5 minutes to quantify CLPM in serum samples, achieving high recoveries but with the requirement of keeping the column at a temperature of 50 ºC and a sample volume of 1 ml (Bahrami et al. 2008).

Rouini et al. developed a rapid and sensitive method on LC with UV detection with a protein precipitation with zinc sulphate method (Rouini et al. 2009).

Other methods have been published with considerably higher sensitivity as carried out by LC/MS-MS instruments (Silvestro et al. 2011, Karazniewicz-Lada et al. 2012).

CLP rapidly disappears from plasma and the relatively low sensitivity achieved with an HPLC/UV instrument do not allow for the quantification of CLP parent compound along with its carboxylic metabolite. A measure of the ratio between them would provide a more direct way to assess CES1 activity and make this method a more powerful tool for PK studies. That may be achieved using a more sensitive methodology such as LC/MS-MS but such instruments are not always readily available in all laboratories and the costs of purchasing and maintaining them are significantly higher than an HPLC/UV instrument.

The aim of this work was to develop a method with relatively simple liquid-liquid extraction phase, that is rapid, reliable, and with enough sensitivity to perform PK studies or TDM when a small sample volume is available. Therefore, the method developed and validated in this section represents a valuable tool in the scope of this thesis which focuses on CES1 enzyme variability.

## 2.3. Optimization and validation of allelic discrimination assays of CES1 gene genetic variants

### 2.3.1. Introduction

To explore the impact of carboxylesterase 1 (CES1) genetic variability on enzyme activity, drug pharmacokinetics and pharmacodynamics, an allelic discrimination (AD) assay was developed, optimized and validated with which to identify the presence of two previously reported naturally occurring genetic variants, namely rs71647872 and rs71647871.

Rs71647871 is a non-synonymous substitution of guanine by adenine in exon 4 causing a glycine in position 143 to be substituted by a glutamic acid. That glycine is key to the hydrolytic efficiency of the native protein because it is part of the oxyanion hole that stabilizes substrate-enzyme intermediates. This variant is thought to reduce the enzyme substrate processing speed (Zhu et al. 2008). Although it is a low frequency variant (1-5%), it has been explored in a number of studies so far and genotyping assays have been developed for its detection, most of them using TaqMan® qPCR based technologies (Nemoda et al. 2009, Tarkiainen et al. 2012, Lewis et al. 2013).

rs71647872 is a thymine deletion within exon 6 resulting in a shift of the reading frame at codon 260. As a consequence, the translated peptide sequence contains 39 altered amino acids after the deletion point followed by an early stop codon, resulting in a 298 amino-acid protein instead of the 567 that constitute the native form. The altered protein displays seriously impaired activity as two of the three amino acids responsible for the hydrolytic activity of the enzyme are missing from the sequence.

As of today only one heterozygous carrier has been reported of this variant (Zhu et al. 2008).

AD assays results are often reported on AD plots where tested samples are grouped in 3 clusters based upon amplification of one or both of the two reporter dyes. Modern qPCR machines generate AD plots automatically but this can prove challenging for low frequency genetic variants like those described in this section because of the absence of 1 or 2 of the clusters. In such cases it may be worth developing artificial positive controls that provide the software with references of known genotype to facilitate genotype calling (van der Straaten and Guchelaar 2013).

In this section optimization and validation of two AD assays for the genotyping of rs71647871 and rs71647872 CES1 genetic variants will be described, along with the development of positive controls for both of them. To avoid confusion from here on rs71647871 and rs71647872 variants will be referred to as Gly143Glu and Arg260fs, respectively.

### 2.3.2. Materials and methods

#### 2.3.2.1. Instrumentation

The thermal cyclers used for optimization and validation of the genotyping assay were GS1 G-Storm (Gene Technologies Ltd, Essex, UK) and ABI 7500 qPCR system (Applied Biosystems, Foster City, California, USA).

Sequencing was performed in-house with an ABI 3730xl DNA Sanger sequencer (Applied Biosystems, Foster City, California, USA). QIA quick PCR purification kit (Qiagen inc., California, USA) was used for purification of PCR products prior to sequencing. BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, California, USA) was used in the sequencing reaction mix. Bidirectional sequencing was performed using primers M13 forward, M13 reverse, Sp6 and T7. Sequences were assembled using Sequencher software v.4.10 (Gene Codes Corporation, Ann Arbor, Michigan, USA).

A Qubit® fluorometer and reagents were used for gDNA and plasmid quantitation (Life Technologies, Paisley, UK).

#### 2.3.2.2. Reagents and kits

Custom TaqMan® primers and probes for both Gly143Glu and Arg260fs, and TaqMan® Universal PCR Master Mix were purchased from Applied Biosystems (Applied Biosystems, Foster City, California, USA), the sequences of primers and probes are those reported in Zhu et al. (2008). The rest of the DNA oligonucleotides used for site-directed mutagenesis (SDM) and amplification of target genome sequences were designed with Primer3 (Untergasser et al. 2012) and ordered from Eurofins (MWG Operon, Ebersberg, Germany).

Topo TA-cloning® kit was obtained from Invitrogen (Invitrogen Inc., Carlsbad, California, USA). QIAquick gel extraction, QIAquick PCR purification and QIA Prep Spin Miniprep® kits were obtained from Qiagen (Qiagen Inc., California, USA).

#### 2.3.2.3. Specimens and controls

45 gDNA samples from healthy volunteers were used for preliminary genotyping testing and further optimization and validation steps of the assay. Purity was assessed using the $A_{260}/A_{280}$ ratio after extraction from whole blood. This work was carried out in conjunction with Lab21 Ltd staff. Lab21 is a molecular diagnostics company where part of this thesis was developed.

However not all genotypes were represented in the resulting AD plots due to the low frequency of both genetic variants. To ensure correct genotype calling by the genotyping software, it was necessary to develop and include positive references.

##### 2.3.2.3.1. Development of controls

The development of artificially synthesized positive controls was necessary to provide the genotype calling software with a reference in the absence of natural mutant carriers. For such purpose it was necessary to first obtain the sequences of interest for both genetic variants and then insert them into plasmid vectors.

For Arg260fs the mutant allele-containing sequence was artificially synthesized by SDM. Primer3 online tool was used to design two sets of primers and two complementary oligonucleotides displaying the mutant nucleotide. Details of the sequences are shown in Table 2-3.

The 27 nucleotide forward and reverse oligonucleotides carrying Arg260fs variant were amplified separately. The products of both reactions were then put together with primers upstream and downstream the mutation and a DNA template to obtain a PCR product with the missing nucleotide. The region spanned 401 base pairs (bp).

After confirmation of the presence of the intended size product by agarose electrophoresis and purification, the segment was cloned into a plasmid vector using the Topo TA-cloning® kit following manufacturer's instructions. Plasmids were purified and stored at -20°C. A plasmid wild-type (WT) was also prepared using a template to serve as WT positive control as well as to construct the heterozygous control. The sequence confirmation procedure will be described later as is shared by both genetic variants.

In the case of the Gly143Glu variant, a preliminary genotyping test was performed on 45 gDNA samples from healthy volunteers. Cycling conditions and PCR reaction mix information can be found in Table 2-4 and Table 2-5.

All samples tested were WT for the Gly143Glu variant except for two that were suspected to be heterozygous, but in the absence of positive controls it could not be confirmed without sequencing them. Therefore a region spanning 316 bp around the substitution position in chromosome 16 was amplified and sequenced in one of the two suspected heterozygous. The amplicon was cloned with Topo TA-cloning® kit and plasmids purified and stored as described above for Arg260fs.

Plasmids obtained after cloning had to be assayed to confirm first integration of the amplicon in the vector and second sequence of the amplicon. A total of 41 plasmid samples were isolated and checked until all 4 target alleles (2 for each of the variants) that would serve as PCR positive controls were obtained. The protocol followed to assess each of the plasmid vectors is summarized below.

Plasmid vectors were incubated with the amplicons and then E.coli competent bacteria assimilated the vectors that carried an ampicillin resistant gene for selection. Colonies were then selected and grown in liquid broth medium supplemented with ampicillin at 37ºC overnight. Tubes were centrifuged, supernatant discarded and the pellet containing the bacteria was subjected to lysis. Vectors were isolated using the QIAprep Spin Miniprep® kit according to manufacturer's instructions.

Restriction analysis with *Eco*RI enzyme was performed on the purified plasmids and an electrophoretic run on a 1% (w/vol) agarose gel confirmed the presence or absence of the correct size insert. In total 6 samples were found carrying a Gly143Glu amplicon and 4 had the Arg260fs one. Figure 2-4 is an example where 3 out of the 6 samples in the electrophoretic run were found to carry the 316 bp amplicon corresponding to the Gly143Glu variant. The segment cut by *Eco*RI is 17 bp bigger than the amplicon because of where the restriction sites lay. A diagram of the vector can be found on Figure 2-5 where *Eco*RI enzyme restriction sites are highlighted and explains the extra bp in the electrophoretic run.

Plasmids that had successfully incorporated the right size amplicon were then sequenced to identify the presence of either the WT or mutant allele for the Gly143Glu variant and to ensure that primer and probe binding regions were free of mutations that would interfere with genotyping assay primer or probes binding. The sequencing was bidirectional with two forward (T7 and M13forward) and two reverse primers (M13reverse and Sp6). Promoters for each of the primers are indicated in the vector diagram in Figure 2-5.

Sequencing results helped identify 2 carriers of each allele of the Gly143Glu variant suitable to be used as PCR positive controls. 1 of each allele of the Arg260fs variant were identified as suitable controls for genotyping PCR.

**Table 2-3 Sequences of oligonucleotides and primers used in the development of Arg260fs and Gly143Glu SNPs positive PCR allelic discrimination controls.**

| SNP | Primer direction | Sequence | Number of nucleotides |
|---|---|---|---|
| Arg260fs | SDM forward | TGAAGAAAGGTGAGTCAAGCCCTTGGC | 27 |
| | SDM reverse | GCCAAGGGCTTGACTCACCTTTCTTCA | 27 |
| | Forward | GAAAACCCAGATGAGAGGTG | 20 |
| | Reverse | TTAGCTACAACCGACCACAA | 20 |
| Gly143Glu | Forward | AGATTGCCTTTTGCAAAGTT | 20 |
| | Reverse | AAGTGCAGTGAGGAGAGTCC | 20 |

**Table 2-4 PCR cycling conditions for allelic discrimination assay**

| Steps | Repetitions | Temperature (ºC) | Time |
|---|---|---|---|
| Pre-read | 1 | 50 | 2 min |
| Initial denaturation | 1 | 95 | 10 min |
| Denaturation | 50 | 92 | 15 sec |
| Primer annealing and extension | | 60 | 1 min |

**Table 2-5 PCR reaction mix for allelic discrimination assay**

| Reagents | Volume per sample |
|---|---|
| Mastermix | 5 µl |
| Primers/probes | 0.25 µl |
| $dH_2O$ | 3.75 µl |
| Sample/water/standard | 1 µl |
| Total volume | 10 µl |

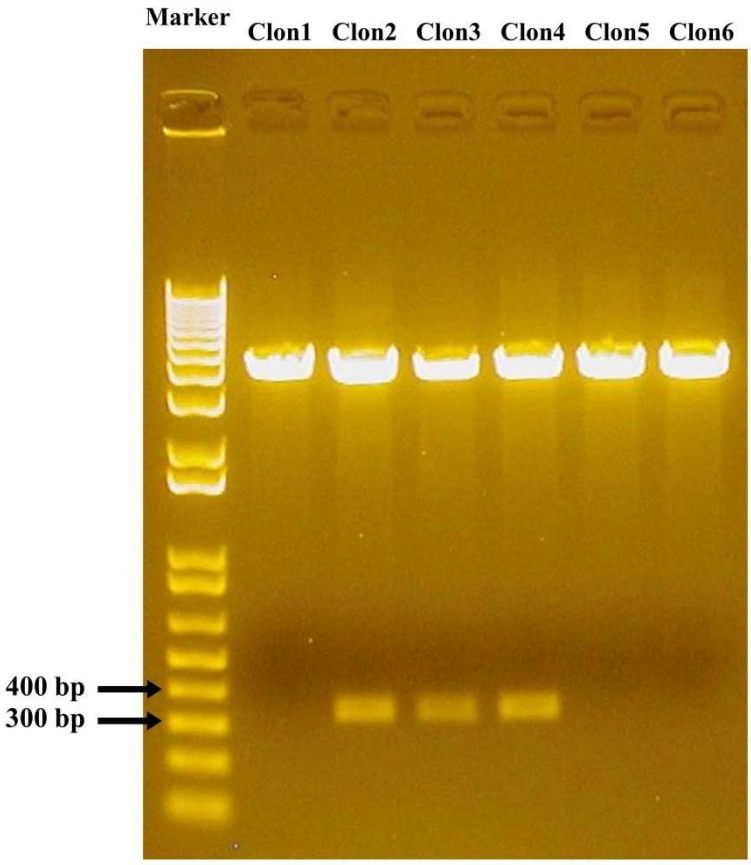


**Figure 2-4 Agarose (1%) gel electrophoresis run.** After enzymatic digestion with restriction enzyme E*co*RI electrophoresis was performed to identify the clones that had integrated the amplicon of interest in their sequence. On the left the marker ladder helped identify the size of the fragments. For Gly143Glu amplicons, sized 316, a band was expected between 300 and 400 bp.

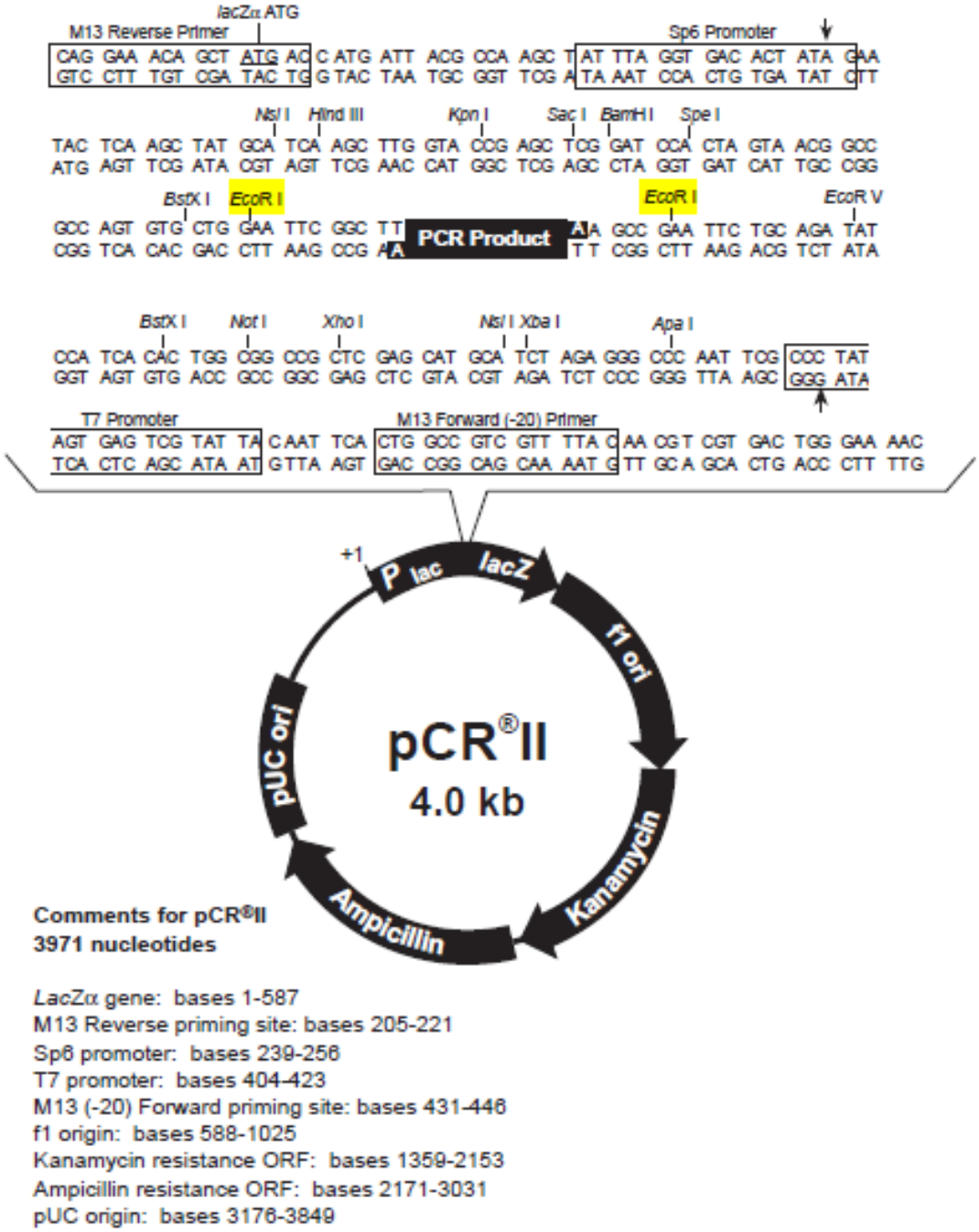


**Figure 2-5. Diagram of the plasmid vector genetic sequence.** Relevant sites on the genome include the amplicon intergration site, the *Eco*R1 cleavage site and the promoter regions for sequencing primers (M13 forward, M13reverse, M7 and Sp6). Image available at http://www.lifetechnologies.com/.

#### 2.3.2.3.2. Controls normalization and copy number

Plasmid controls of Arg260fs and Gly143Glu genetic variants obtained by cloning as described in the previous section were quantified with Qubit® and subsequently diluted and normalized in order to create heterozygous controls with equivalent ratios of mutant and WT alleles.

A single copy of human gDNA contains approximately 3,400,000,000 bp and the plasmids used for this work are made of 4000 bp. Equal concentrations of these two molecules contain a very different number of gene copies. To be able to compare between amounts of gDNA samples and plasmid controls it was necessary to relate to copy number instead of concentration.

### 2.3.2.4. Assay optimization

Assay optimal annealing temperature and sensitivity were assessed and optimized once the positive controls for both genetic variants were ready. Sensitivity was defined by the limit of detection (LOD); the amount of template DNA that the technique can specifically detect and amplify.

To select the optimal annealing temperature an experiment was designed where DNA copy number was kept constant across a plate that was subjected to a range of annealing temperatures in a GS1 G-Storm thermal cycler.

To keep the experiment as homogeneous as possible an initial solution containing PCR Mastermix, primers, probes and PCR-grade water was prepared with the same reagent amounts detailed in Table 2-5 and then distributed in three separate tubes where equivalent and normalized amounts of plasmid representing all three possible

genotypes (homozygous WT, heterozygous, homozygous mutant) were used. 10 µL were added to each well across a 96-well plate in a total of 12 wells per genotype. Negative controls were also added to the plate.

Once the plate was ready it was covered with a septa cover and inserted in an ABI 7500 for a pre-read run at 50ºC to obtain a reading of baseline fluorescence. The plate was then transferred to the G-Storm device, programmed to incubate the plate on a cycle of temperatures similar to the one described in Table 2-4 except that the annealing temperatures differed slightly between plate columns (1-12) ranging from 55.1ºC to 68.2ºC.

After completion of the amplification the plate was returned to the ABI 7500 for a final reading of fluorescence. The assay looked robust across temperatures. The optimal range fell between 58.9ºC and 64.0ºC. Three temperatures were chosen within the optimal range to further optimize assay behaviour and sensitivity. Experiments performed in the ABI 7500 at 60ºC, 62ºC and 64ºC revealed that the temperature that allowed greater sensitivity without compromising specificity was 60ºC.

Finally, a range of copy numbers of all three possible genotypes was assessed at the designated temperature of 60ºC to determine LOD for each of the genetic variants. Arg260fs and Gly143Glu assays achieved LODs corresponding to 5 ng/µL and 1 ng/µL of gDNA, respectively.

Once annealing temperature and LOD had been optimized, validation experiments for both genetic variants genotyping assays were performed.

### 2.3.2.5. Assay validation

To validate the genotyping assays for Arg260fs and Gly143Glu four experiments were carried out for each of the variants in order to assure specificity, accuracy, sensitivity and reproducibility.

Both assays were validated to discriminate test samples in 3 clusters: homozygous WT, heterozygous and homozygous mutant, with a LOD of 5 ng/µL for Arg260fs and 1 ng/µL for Gly143Glu.

Details of cycle conditions and reaction volumes were similar to those listed in the tables in the section above.

The four validation experiments for each of the two genetic variants were performed as described below.

- Experiment 1: 45 gDNA samples were tested with negative controls and positive controls representing all 3 genotypes.

- Experiment 2: 10 of the previously tested samples were tested again in triplicate with negative controls and positive controls representing all 3 genotypes.

- Experiment 3: The same 10 same samples tested in the previous experiment were tested again, this time on a different ABI 7500 instrument and by a different operator.

- Experiment 4: Dilution series of the three genotypes including copy number above and below the LOD were tested. Plasmid positive controls were used for Arg260fs variant. In the case of Gly143Glu, gDNA samples previously identified as WT and heterozygous were used to prepare the dilution series; for the homozygous mutant a plasmid dilution was used. For homogeneity purposes, copy number was used to assess LOD.

### 2.3.3. Results of the assays validation

During the validation experiments the optimized assays specificity was proven by their ability to discriminate between alleles that differed in just one nucleotide and assigning samples to one of the three possible genotypes: homozygous WT, heterozygous, and homozygous mutant. Accuracy was shown by successfully assigning the correct genotype to positive controls.

In Figure 2-6 there is an example of an allelic AD plot obtained after genotyping 45 healthy volunteers for Arg260fs. The two samples identified as mutant homozygous and heterozygous are the plasmid references. All individuals of the cohort were identified as WT for Arg260fs. For Gly143Glu, all individuals were also WT except two that were heterozygous.

10 of the 45 gDNA samples that had been tested in experiment 1 were re-tested in triplicate in experiment 2 for both mutations. In both cases the concordance with the first reported genotype as well as between repeats of the same experiment was 100%, demonstrating intra as well as inter-assay precision.

Moreover, experiment 3 proved that both assays were able to cope with variable test conditions by successfully displaying instrument-to-instrument, operator-to-operator and run-to-run reproducibility. The assays were also proved to cope with variation in sample quality by reproducing analogous results for samples after several freeze-thaw cycles.

A series of dilutions were performed on samples and control materials displaying all genotypes for both genetic variants to assess sensitivity. The assay proved able to discriminate the correct genotype in samples with a concentration of 5 ng/µL and above for Arg260fs and as low as 1 ng/µL for Gly143Glu, the resulting AD plot for the latter can be seen on Figure 2-7.

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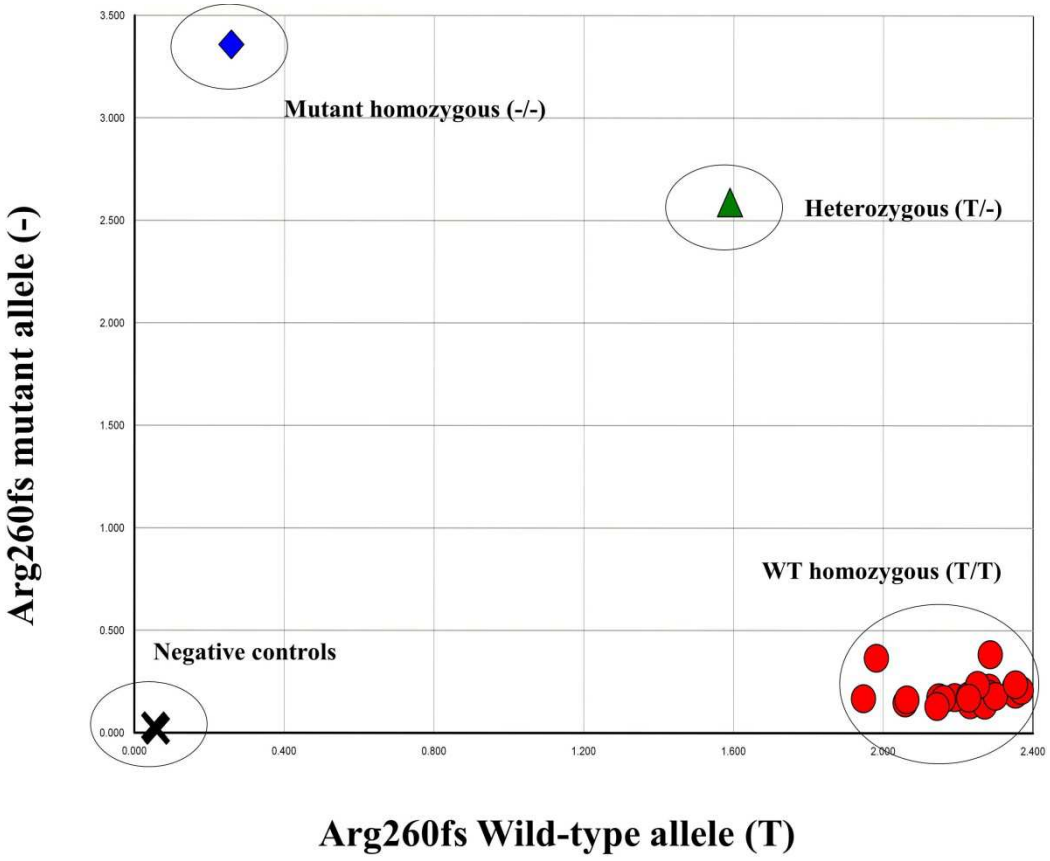


**Figure 2-6 Arg260fs Allelic discrimination plot.** Genotyping was performed on 45 gDNA samples from healthy volunteers together with positive and negative controls. WT genotype samples are represented as a red circle, heterozygous genotype samples are represented as a green triangle, mutant genotype samples are represented as a blue diamond. Negative controls are represented as black crosses.

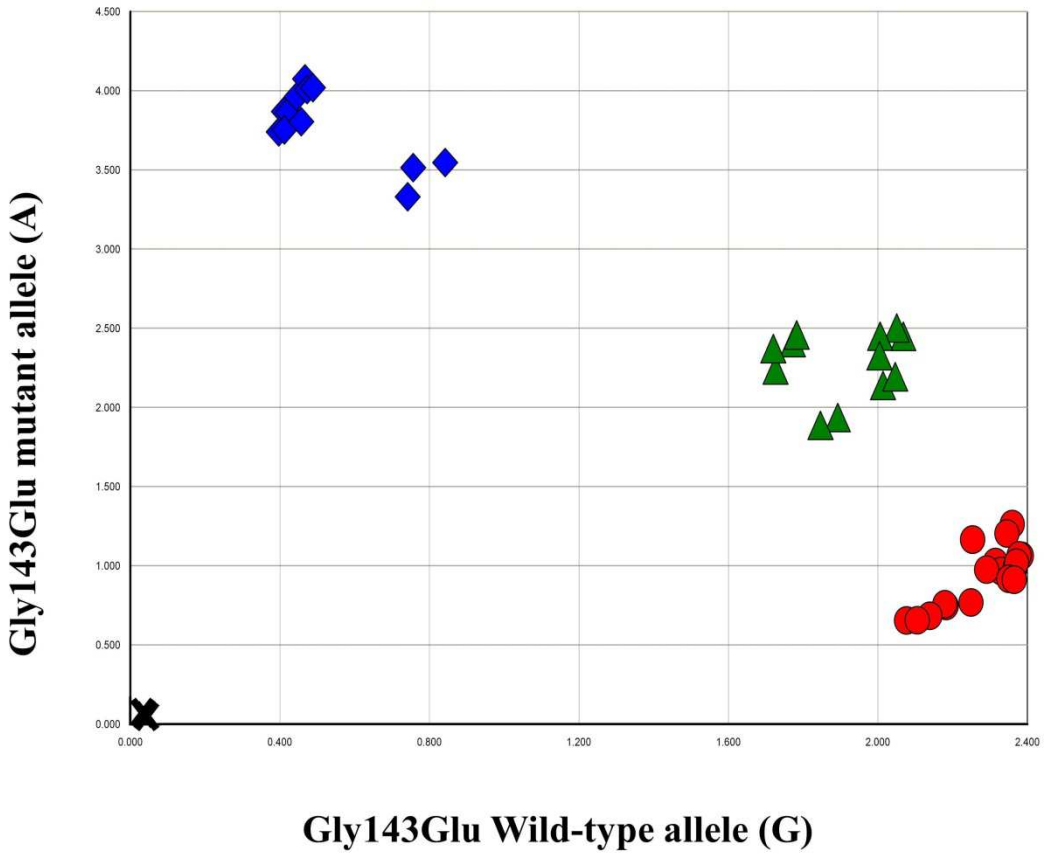


**Figure 2-7 Gly143Glu Allelic discrimination plot.** Genotyping was performed on a series of dilutions of positive controls together with negative controls. WT genotype samples are represented as a red circle, heterozygous genotype samples are represented as a green triangle, mutant genotype samples are represented as a blue diamond. Negative controls are represented as black crosses.

### 2.3.4. Discussion

The idea to develop and validate the method described above was based on the potential value to accurately detect individuals of the population that carry genetic variants of the CES1 gene that may affect the efficiency by which enzyme substrates, and more specifically to this work, pharmacologically active compounds, get processed and the effect that this may have on treatment outcome.

The validation of the method confirmed the assays' ability to accurately assign a genotype call to unknown gDNA specimens for Gly143Glu and Arg260fs genetic variants.

The specificity of the assay was assured by comparison of unknown samples with standards that had been created by cloning. 100% concordant results confirmed inter and intra-assay precision after multiple testing on the same and different days. Robustness was shown by the ability of the assay to cope with variation in operator, instrument and sample quality.

One of the strengths of the method described above is the low sensitivity achieved which may be especially useful when testing DNA extracted from buccal swabs, forensic samples or any other context where little sample is available or the quality of the DNA may have been compromised.

Several qPCR methods have been developed to characterize CES1 genotype for Gly143Glu SNP, even after a genomic assessment of CES1 gene that claimed that regular SNP genotyping assays would be unable to detect mutant homozygosity because of the high homology between the functional isoform of the gene (CES1A1),

the low transcription isoform (CES1A2) and the CES1A3 pseudogene (Zhu et al. 2012). This report raises the interesting point that genetic polymorphisms located in the functional isoform (CES1A1) that accounts for about 98% of the mature protein have the potential of exerting a bigger effect than those that appear in the CES1A2 isoform whose transcription rate is 2% of the total amount of mature protein. They identified Gly143Glu as a variant located on the CES1A1 isoform which may explain why this variant has been repeatedly reported as a genetic marker with great effect on the pharmacokinetics of compounds such as CLP and oseltamivir (Tarkiainen et al. 2012, Lewis et al. 2013). It would be interesting in any case for future assessment of CES1 variants to identify genomic location, since the isoform where they occur may contribute to the strength of the effect.

On the other hand the Arg260fs variant was found to be located on CES1A2 and assessment of the independent effect of this variant remains unexplored until another carrier is found. The only ever reported carrier was simultaneously a Gly143Glu carrier (Zhu et al. 2008).

In conclusion, the assay developed in this section for genotyping of Gly143Glu, is a relatively easy and cheap way to detect the Glu allele carriers and it will serve our purposes. Gly143Glu is a low frequency variant with a MAF of up to 4% in Caucasians, therefore separating mutant carriers further is only likely to reduce the power of our studies. In any case, if considered worthwhile for the framework of the thesis, the samples identified as heterozygous with this method could be subsequently sequenced to confirm it.

# CHAPTER 3

# Effect of CES1, CYP2C9 and CYP2C19 genetic variability on clopidogrel antiplatelet efficacy

# Chapter 3: Effect of CES1, CYP2C9 and CYP2C19 genetic variability on clopidogrel antiplatelet efficacy

## 3.1. Introduction

Cardiovascular disease remains the first cause of mortality worldwide despite a considerable reduction over the last decades thanks to identification of risk factors, prevention campaigns and improvement of pharmacological treatments (Alwan 2011).

Cardiovascular disease is a complex, multifactorial condition whose presentation, progression and severity depends on the interaction of genetic, environmental, physio-pathological and lifestyle factors over time. These contribute to the development of aetiologies such as atherosclerosis, hypertension and hypercholesterolemia which may eventually lead to acute episodes. The damage caused by such episodes depends upon the anatomical region and the extent of tissue affected. The most severe forms of cardiovascular disease are cerebrovascular accidents and acute coronary syndromes (ACS) affecting blood supply to the brain and myocardial muscle, respectively.

ST-segment elevation myocardial infarction (STEMI), non ST-segment elevation myocardial infarction (NSTEMI) and unstable angina are some of the most prevalent forms of ACS. They require prompt interventions that usually combine pharmacological treatment and physical procedures to restore perfusion of the cardiac muscle, such as coronary artery bypass grafting (CABG) and percutaneous coronary intervention (PCI).

Administration of dual-therapy with clopidogrel (CLP) and aspirin has been shown to reduce both mortality within the next year after a PCI (Mehta et al. 2001) and the risk of stent thrombosis, a rare but dangerous side effect of stent implants (Iakovou et al. 2005).

CLP is administered orally and is rapidly absorbed and transported into the portal circulation. Evidence suggests that the absorption process may involve the P-glycoprotein intestinal efflux pump encoded by ABCB1 gene (Taubert et al. 2006). Following absorption CLP is inactivated by carboxylesterase 1 (CES1), leaving only about 15% of the parent compound to enter the activation cascade. The formation of the active metabolite involves a two-step process mediated by cytochrome P450 enzymes (CYP P450). The first step yields the intermediate 2-oxo-clopidogrel which is further oxidized to the active thiol compound in the second step (Kazui et al. 2010). Both intermediate and active metabolites are further inactivated by CES1 (Zhu et al. 2013) (Details in Figure 3-1).

The mechanism of action of the active metabolite of CLP is an irreversible inhibition of the $P_2Y_{12}$ receptor expressed on the platelet membrane. As a result, platelets are unable to engage in ADP-dependent aggregation for the rest of their lifespan (7-10 days). There is no antidote for this effect, it is necessary to generate new platelets or to undergo a platelet transfusion to produce efficacious platelet aggregation again.

Nevertheless, 20% to 40% of patients are resistant or poor-responders to treatment with CLP (Brandt et al. 2007). Among the factors known to contribute to the well reported inter-individual variability in CLP treatment response there are demographic, clinical, iatrogenic and genetic causes including age, body mass index (BMI), diabetes mellitus, renal failure (creatinine > 1.5 mg/dl), reduced ventricular function and drug-drug interactions (DDIs) (Trenk et al. 2012).

Polypharmacy is frequent in cardiovascular patients. CLP is commonly co-administered with agents such as statins, β-blockers, angiotensin converting enzyme inhibitors (ACEIs) and antidiabetics among others. DDIs mediated by some of the CYPs involved in the two-step activation pathway of CLP have been reported. Among the better covered are the interactions between CLP and the proton pump inhibitor omeprazole via inhibition of CYP2C19 (Gilard et al. 2006), the calcium-channel antagonist verapamil via CYP3A4 inhibition (Siller-Matula et al. 2008), and atorvastatin via CYP3A4 inhibition, although there are conflicting data regarding the latter (Lau et al. 2003).

In addition to DDIs, genetic variability of the genes encoding for enzymes and receptors relating to CLP metabolic steps and targets has been reported. The focus has been mainly on the CYP-mediated two-step CLP activation cascade in the liver.

CYP2C19 has shown the strongest effect among all, and carriers of CYP2C19*2 or CYP2C19*17 alleles are referred to as poor and ultra-rapid metabolizers, respectively. In this regard, the U.S. Food and drug administration (FDA) included a warning in the CLP label in 2010 advising that carrying a CYP2C19 reduced function allele was associated with a significantly increased risk of major adverse cardiovascular events and that genetic tests were available to determine a patients' CYP2C19 genotype. However, there was not a consensus within the scientific community with some suggesting that the level of evidence was not strong enough to justify the FDA decision (Nissen 2011).

Unlike the above described CYP P450 enzymes, CES1 genetic variability effects on CLP metabolism have not yet been investigated in depth. However, naturally occurring genetic polymorphisms exist in the CES1 gene that may affect the activity of the enzyme, which could in turn modify CLP metabolic profile and ultimately have an impact on its therapeutic effect and adverse reactions.

Over the 15 years since its commercialization a great deal of literature has become available regarding CLP treatment variability. Yet due to the complexity of both disease development and pharmacological response, the underlying mechanisms that determine treatment variability remain poorly understood. Nevertheless, evidence suggests that low availability of CLP active metabolite translates into low treatment efficacy (Heestermans 2008). Therefore, understanding the factors that influence CLP metabolic profile is a key factor for understanding the determinants of treatment variability which in turn may enable clinicians to improve CLP treatment responsiveness.

In this chapter the focus is on the role of CES1 genetic variability on CLP metabolism. The hypothesis is that genetic variants of CES1 may affect the catalytic efficiency or expression of the enzyme and have an effect on CLP metabolic profile, which in turn might impact CLP antiplatelet activity. Two CYP2C19 and two CYP2C9 genetic variants were also explored in the course of this work.

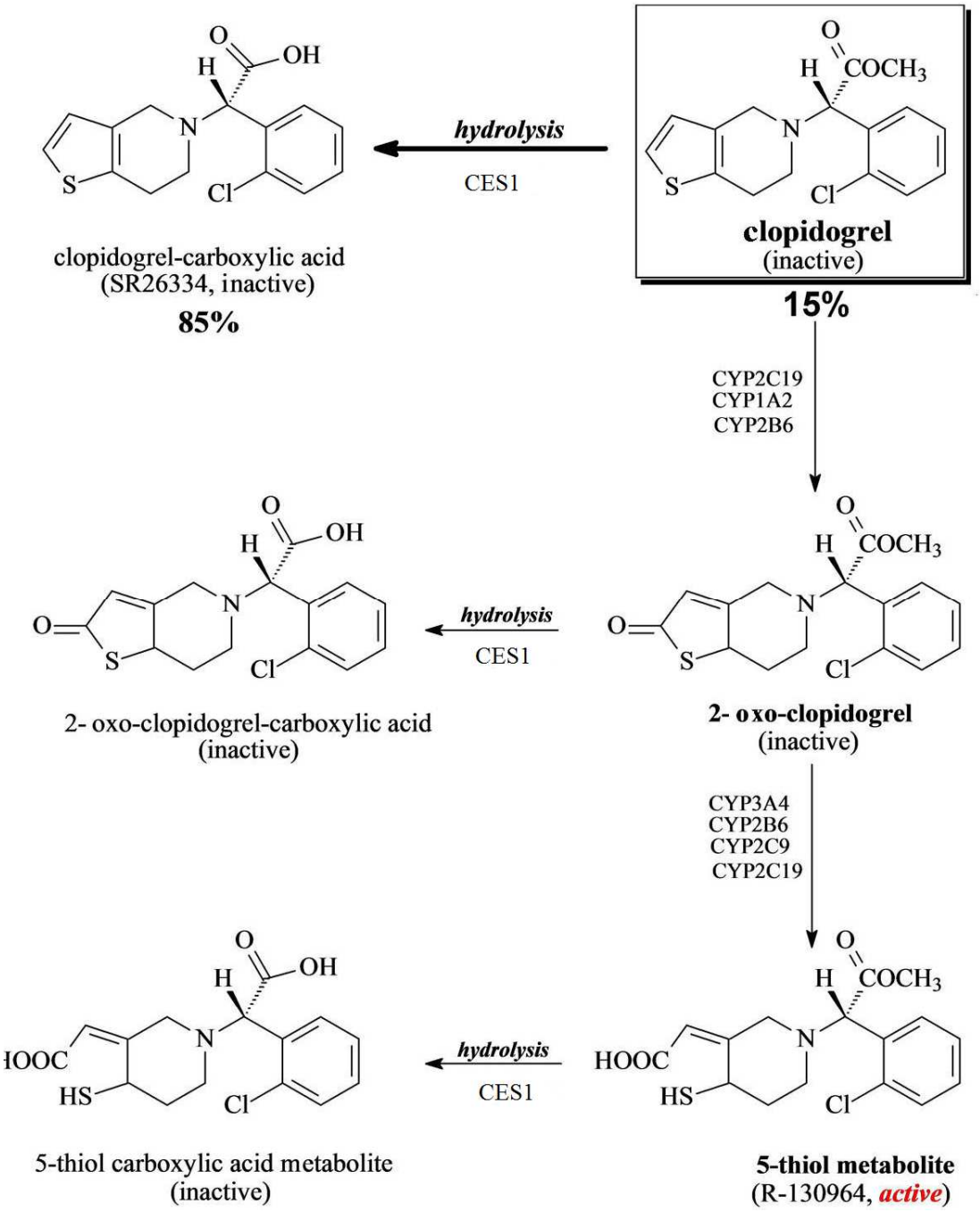


**Figure 3-1 CLP metabolic pathway:** CLP biotransformation from the inactive prodrug to the active thiol metabolite via CYP P450 oxidation and the CES1-mediated hydrolytic reaction that yields CLP and its metabolites inactive. (Modified image from Zhu et al. (2013)).

## 3.2. Methods

### 3.2.1. Clinical study

The PhACS study (Pharmacogenetics of Acute Coronary Syndrome) was a prospective, observational, multi-centre study led by the Department of Molecular and Clinical Pharmacology at the University of Liverpool, to study the pharmacogenomics of ACS treatment. The three recruiting clinical centres were the Royal Liverpool University Hospital, Blackpool Victoria Hospital and Liverpool Heart and Chest Hospital, United Kingdom.

The study objectives were to investigate the effect of genetic variants on pharmacokinetics, pharmacodynamic endpoints, mortality, cardiovascular events and bleeding in patients treated with clopidogrel after an acute coronary syndrome.

The inclusion criterion was a main diagnosis of acute coronary syndrome in index hospital admission. Clinically-related exclusion criteria included suffering from a pathological condition other than ACS likely to account for symptoms or troponin rise, or that may lead to non-cardiac death within 1 year. ST-elevation myocardial infarction sufferers were also excluded.

The study was designed in three visits, at baseline, 1 month and 12 months. During these visits plasma and blood for genetics and platelet function were obtained. Demographic and clinical data were collected during the baseline visit. Diabetes, smoking status and treatment compliance were determined by self-report.

All study protocols were approved by the respective institutional review boards at the University of Liverpool, Royal Liverpool University Hospital, Blackpool Victoria Hospital, Liverpool Heart and Chest Hospital and adhered to the principles of the Declaration of Helsinki. Written informed consent was signed by all participants.

### 3.2.2. Study population

The focus of this chapter is on CLP activity; therefore for the purpose of this work a subset of 187 patients of the PhACS trial cohort was selected. Members of this subcohort had been treated with 75 mg of CLP daily before having platelet aggregation measured by the Multiplate® instrument (Roche, Basel, Switzerland) at visit 2 (1 month).

46 subjects within the pre-selected 187 group were included in a pharmacokinetic study to explore the impact of CES1 genotype on plasma concentrations of clopidogrel carboxylic metabolite (CLPM). Plasma specimens had been collected within 3 hours of CLP dosing. Plasma was only collected at one of the clinical centres (The Royal Liverpool University Hospital).

### 3.2.3. Platelet function testing

The Multiplate® instrument uses impedance aggregometry technology which is based on the principle that platelets are non-thrombogenic in their resting state but they can stick to surfaces when they get activated by biochemical signals like adenosine diphosphate (ADP) released following vascular injury.

The Multiplate® system quantifies platelet aggregation in whole blood by measuring the reduction in conductivity between two electrodes when active platelets attach to them. CLP irreversibly inhibits platelet surface $P_2Y_{12}$ receptors that bind ADP, interfering with platelet activation.

Whole blood was drawn from all 187 participants at 1 month and, after stimulation with ADP, tested for platelet aggregation by the Multiplate® instrument. Results are expressed in arbitrary units per minute (AU*min) that take into consideration intensity and velocity of aggregation. The Multiplate® system total run time is 6 minutes.

### 3.2.4. Genotyping

DNA was extracted from whole blood by researchers at the Wolfson Centre for Personalized Medicine. Purity was assessed using the $A_{260}/A_{280}$ ratio.

DNA samples that were of interest for this sub-study were aliquoted. Normalization was carried out with the Biomek® NXP Laboratory Automation Workstation (Beckman Coulter, Fullerton, California, USA). Dilution of DNA was performed using 1% Tris-EDTA buffer (Trisaminomethane-Ethylenediaminetetraacetic Acid) as a diluent, which was prepared solubilizing Tris-EDTA powder (Sigma-Aldrich, St Louis, Missouri, USA) in deionised water. The final concentration of DNA for each sample was 20 ng/µl.

An ABI 7300 real-time polymerase chain reaction (qPCR) platform and off-the-shelf TaqMan® SNP Genotyping assays (Applied Biosystems, Foster City, California, USA) were used for DNA genotyping. Tests were performed in 386-well plates. The reaction volume was 5 µl of which 2.5 µl was TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA), 0.125 µl was a mixture of probes and primers specific for each SNP, and 2.375 µl was deionised water. Approximately 10% of the samples were genotyped in duplicate.

The choice of CES1 SNPs included four that had been shown in previous studies to exert a phenotypic effect (rs71647871, rs71647872, rs2244613 and rs3815583) and two new variants (rs62028647 and rs3826190) that were identified by the *in silico* tools SIFT (Kumar et al. 2009) and Polyphen-2 (Adzhubei et al. 2010). These online software packages attempt to predict how damaging an amino acid substitution might be for protein function based on the degree of conservation across protein relatives. For SNPs selected this way, only those found with a minor allele frequency (MAF) of 5% or above in Caucasian populations were included. SNP frequencies were obtained from the National Center for Biotechnology Information SNP database (http://www.ncbi.nlm.nih.gov/snp Last accessed Sept2014).

Most samples had previously been genotyped at the Wolfson Centre for CYP2C19 (*2,*17) and CYP2C9 (*2, *3) genes following the same protocol as described above for the CES1 variants. All the SNPs genotyped during the course of this work are listed in Table 3-1.

**Table 3-1 CES1 and CYP P450 SNPs included in the pharmacogenetic assessment**

| Accession Number | Gene | Ch. | Effect | Nucleotide change | MAF |
|---|---|---|---|---|---|
| rs71647871 | CES1 | 16 | p.G143E | G/A | No data |
| rs71647872 | CES1 | 16 | p.R260- | T/- | No data |
| rs62028647 | CES1 | 16 | p.S83L | G/A | 0.37 |
| rs3826190 | CES1 | 16 | p.G18V | G/T | 0.3 |
| rs2244613 | CES1 | 16 | Intronic | A/C | 0.15 |
| rs3815583 | CES1 | 16 | UTR-5 region | T/G | 0.15 |
| rs1799853 | CYP2C9 (*2) | 10 | p.R144C | C/T | 0.1 |
| rs1057910 | CYP2C9 (*3) | 10 | p.I359L | A/C | 0.06 |
| rs4244285 | CYP2C19 (*2) | 10 | p.P227P | G/A | 0.16 |
| rs12248560 | CYP2C19 (*17) | 10 | Intronic | C/T | 0.22 |

Ch.: Chromosome. MAF: Minor allele frequency. NCBI SNP database was accessed in September 2014.

### 3.2.5. Determination of plasma metabolite concentrations

Development and validation of the methodology used to quantify CLPM has been explained in detail in section 2.2. Briefly, measurement was carried out with a High Performance Liquid Chromatography technique with ultraviolet detection (HPLC/UV) instrument at a detection wavelength of 200 nm. The column employed was a Hypersyl gold 150x4.6 mm and 5 µm of particle size (Thermo Scientific Inc. Waltham, Massachusetts, USA). A gradient was applied with varying concentrations of organic solvent (acetonitrile:water, 90:10) and aqueous solvent ($KH_2PO_4$ 10 mM with 200 µl orthophosphoric acid (OFA), pH =3.2). Flow rate was 1 ml/min. Liquid-liquid extraction was performed with ethyl acetate on 200 µL volume of plasma. The limit of detection (LOD) was 78 ng/ml. The method was linear between 78 and 10,000 ng/ml.

### 3.2.6. Data analysis

IBM SPSS® for Windows®, version 22 (SPSS, Illinois, USA) was used for statistical analysis. Figures were produced with Graphpad Prism v. 5.01 (La Jolla, California, USA). Normality of continuous variables was assessed by the Shapiro-Wilk test, as well as from observations of histograms and quantile-quantile (Q-Q) plots.

Chi-squared tests were performed to explore significant deviations from Hardy-Weinberg equilibrium of genetic variants. Analysis of variance (ANOVA) was performed to compare mean response variables between demographic and clinical categorical variables as well as mean differences between SNP genotypes. Linkage disequilibrium between SNPs was explored using Haploview software (Barrett et al. 2005).

Linear regression was performed in order to identify significant correlations between continuous variables. General linear models were applied for multifactorial analyses. P values to an alpha of less than 0.05 were considered statistically significant.

## 3.3. Results

### 3.3.1. Patient demographics

A total of 187 subjects were included in the pharmacogenetic assessment. Their demographic and clinical data are summarized in Table 3-2. About half of the cohort was above 65 years of age.51 study subjects were female (27%), 136 were male (73%). The population was almost completely of Caucasian ancestry, except 2 individuals of black African ancestry. 40% of the subjects reported being non-smokers, 22% current smokers and 32% former smokers. 17% of the participants reported suffering from diabetes.

As expected of a pragmatic trial on ACS patients, concomitant drugs were numerous. This cohort was treated with a mean of 14 other compounds in addition to CLP. The most frequently prescribed were statins (99%), ACEIs (79%), ß-blockers (89%) and nitrates (57%). None of the patients was being treated with GPIIb/IIIa antagonists which can interfere with the Multiplate® ADP test.

**Table 3-2 Study cohort demographic data**

| Demographic and clinical data | | |
|---|---|---|
| Age (years) | Age < 65 | 98 (52) |
| | Age ≥ 65 | 89 (48) |
| Sex | Female (n, %) | 51 (27) |
| | Male (n, %) | 136 (73) |
| Ethnicity | Black African (n, %) | 2 (1) |
| | White English (n, %) | 185 (99) |
| Smoking status | Non-smoker (n, %) | 74 (40) |
| | Current smoker (n, %) | 41 (22) |
| | Former smoke (n, %) | 61 (32) |
| | Unknown (n, %) | 11 (6) |
| Diabetes | Diabetic (n, %) | 31 (17) |
| | Non-diabetic (n, %) | 151 (81) |
| | Unknown (n, %) | 5 (2) |

### 3.3.2. SNP frequencies and linkage disequilibrium

Our target population of 187 patients was genotyped for a total of 10 SNPs located in CES1, CYP2C9 and CYP2C19 genes.

SNPs rs71786472 and rs3826190 in the CES1 gene were monomorphic and no further analysis was performed on them. The remaining 8 SNPs did not display significant deviations from Hardy-Weinberg equilibrium except for rs3815583, which was excluded from the analysis. For all other SNPs the mean calling rate was 95% and the mean genotype concordance rate in a subset of 10% duplicate samples was 100%.

Genotype and allele frequencies of the SNPs included in this study are shown in Table 3-3. Minor allele frequencies (MAF) do not differ greatly with those previously reported in Caucasian populations (Table 3-1).

Linkage disequilibrium (LD), the probability that two alleles are inherited together, was explored using Haploview. The LD plot obtained is shown in Figure 3-2. Two of the CES1 SNPs (rs62028647 and rs2244613) were in high LD. As expected, SNPs in CYP2C9 and CYP2C19 SNPs also appear highly correlated with each other because both these genes are located on chromosome 10.

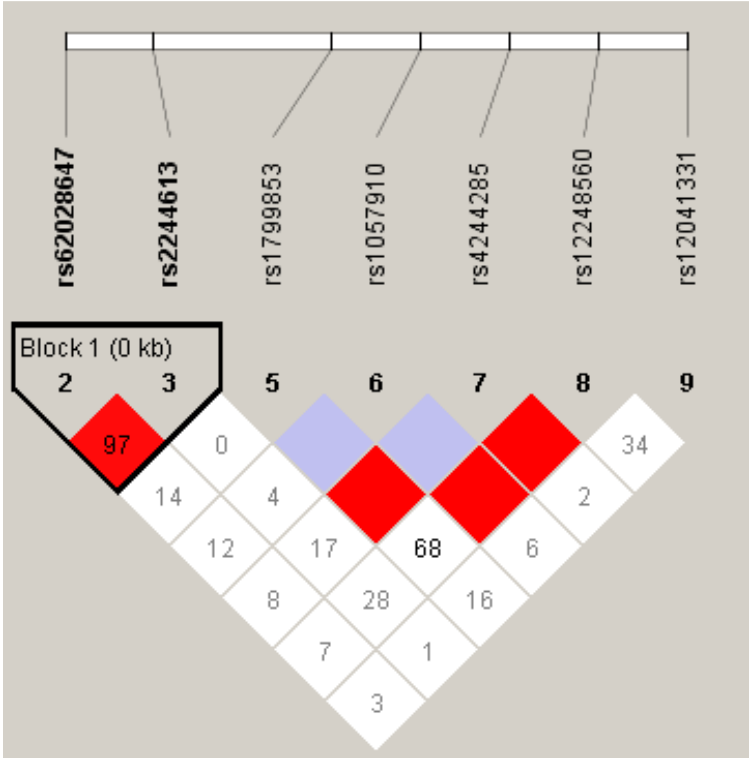


**Figure 3-2 Linkage disequilibrium (LD) plot obtained with Haploview software.** LD measures the probability that two SNPs are inherited together. Red boxes indicate high LD between SNPs.

**Table 3-3 SNPs genotyping results, allele frequencies and Hardy-Weinberg equilibrium**

| Accession Number | N | Genotype Calling rate | Genotype count | | | Genotype Frequency | | | MAF | H-W P value |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $A_1/A_1$ | $A_1/A_2$ | $A_2/A_2$ | $A_1/A_1$ | $A_1/A_2$ | $A_2/A_2$ | | |
| rs71647871 | 177 | 95% | 171 | 6 | 0 | 0.97 | 0.03 | 0.00 | 0.02 | 0.82 |
| rs62028647 | 174 | 93% | 70 | 83 | 21 | 0.40 | 0.48 | 0.12 | 0.36 | 0.63 |
| rs2244613 | 178 | 95% | 107 | 65 | 6 | 0.60 | 0.37 | 0.03 | 0.22 | 0.30 |
| rs3815583 | 115 | 62% | 64 | 49 | 2 | 0.56 | 0.43 | 0.02 | 0.23 | 0.03 |
| rs1799853 | 179 | 96% | 126 | 46 | 7 | 0.70 | 0.26 | 0.04 | 0.17 | 0.29 |
| rs1057910 | 179 | 96% | 164 | 14 | 1 | 0.92 | 0.08 | 0.01 | 0.04 | 0.26 |
| rs4244285 | 175 | 94% | 139 | 35 | 1 | 0.79 | 0.20 | 0.01 | 0.11 | 0.44 |
| rs12248560 | 176 | 94% | 104 | 63 | 9 | 0.59 | 0.36 | 0.05 | 0.23 | 0.89 |

N: Number of samples. $A_1/A_2$: Main and minor alleles. MAF: Minor allele frequency. H-W: Hardy-Weinberg equilibrium. P value calculated with the Chi squared test.

### 3.3.3. Impact of demographic, clinical and genetic factors on platelet aggregation

Data obtained from measuring platelet aggregation in CLP treated subjects was assessed for normality by Shapiro-Wilk test as well as visual evaluation of histograms and Q-Q plots. Neither raw aggregation nor log-transformed aggregation adjusted to a Gaussian distribution. However, a square root transformation of the data satisfied both mathematical and visual assessments of normality and was ultimately used for data analysis. Histograms can be found in Figure 3-3.

The impact of age, sex, diabetes and smoking status on CLP-induced platelet aggregation is depicted in Figure 3-4. The impact of CES1, CYP2C9 and CYP2C19 SNPs on CLP-induced platelet aggregation is depicted in Figure 3-5. Genotypes carried by 5 or less individuals were grouped together with heterozygous genotypes in the figures and the analysis.

Univariate analysis of variance was performed to identify significant differences between means but none of the factors tested in this study appeared individually associated with platelet aggregation. Variables with a P value of less than 0.200 were included in a multifactorial analysis meaning smoking status was selected as a correcting factor (0.100). After the correction C allele carriers of the rs2244613 SNP significantly correlated with platelet aggregation (P=0.034). The results of both univariate and multivariate analysis can be found in Table 3-4.

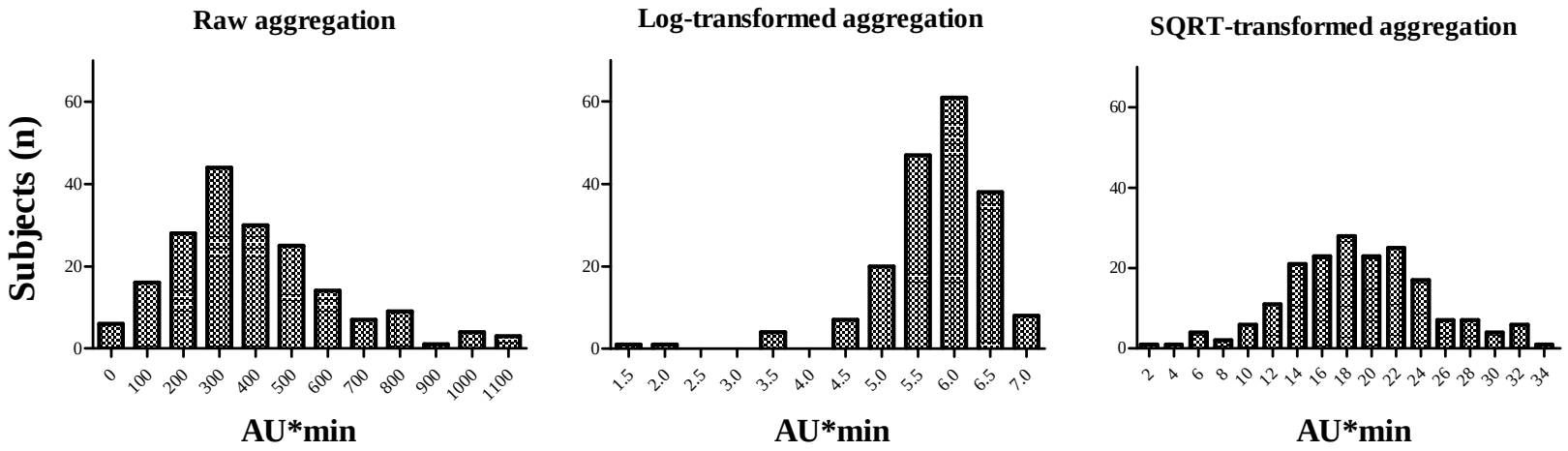


**Figure 3-3 Histogram of platelet aggregation data measured in arbitrary units per minute (AU*min) against subject count for assessment of normality.**

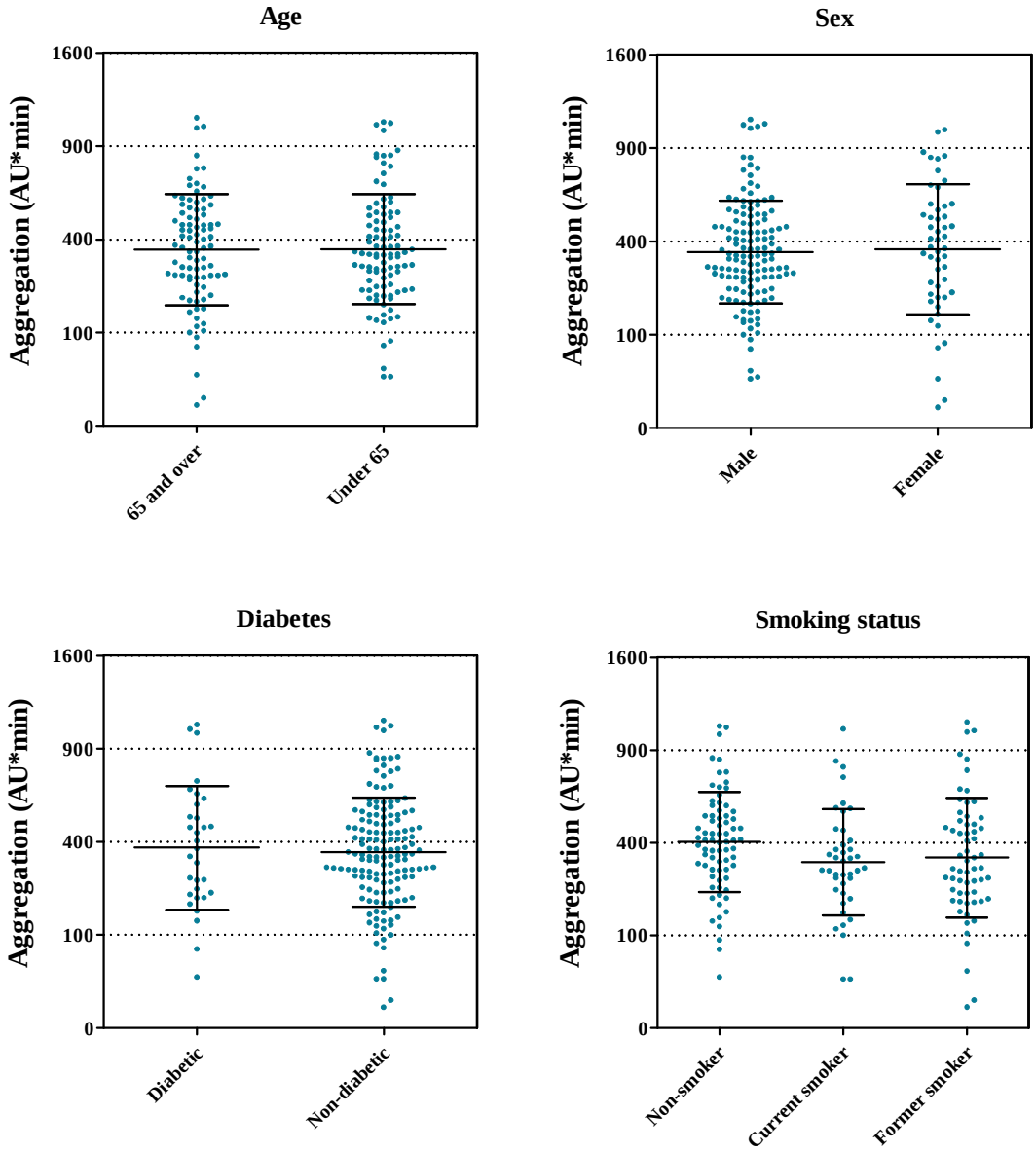


**Figure 3-4 Impact of demographic and clinical factors on CLP-induced platelet aggregation.** Scatter dot plots of platelet aggregation data by age, sex, diabetes and smoking status. The lines represent the mean and the bars the standard deviation (SD). Y axis is displayed in a back transformed SQRT-scale.

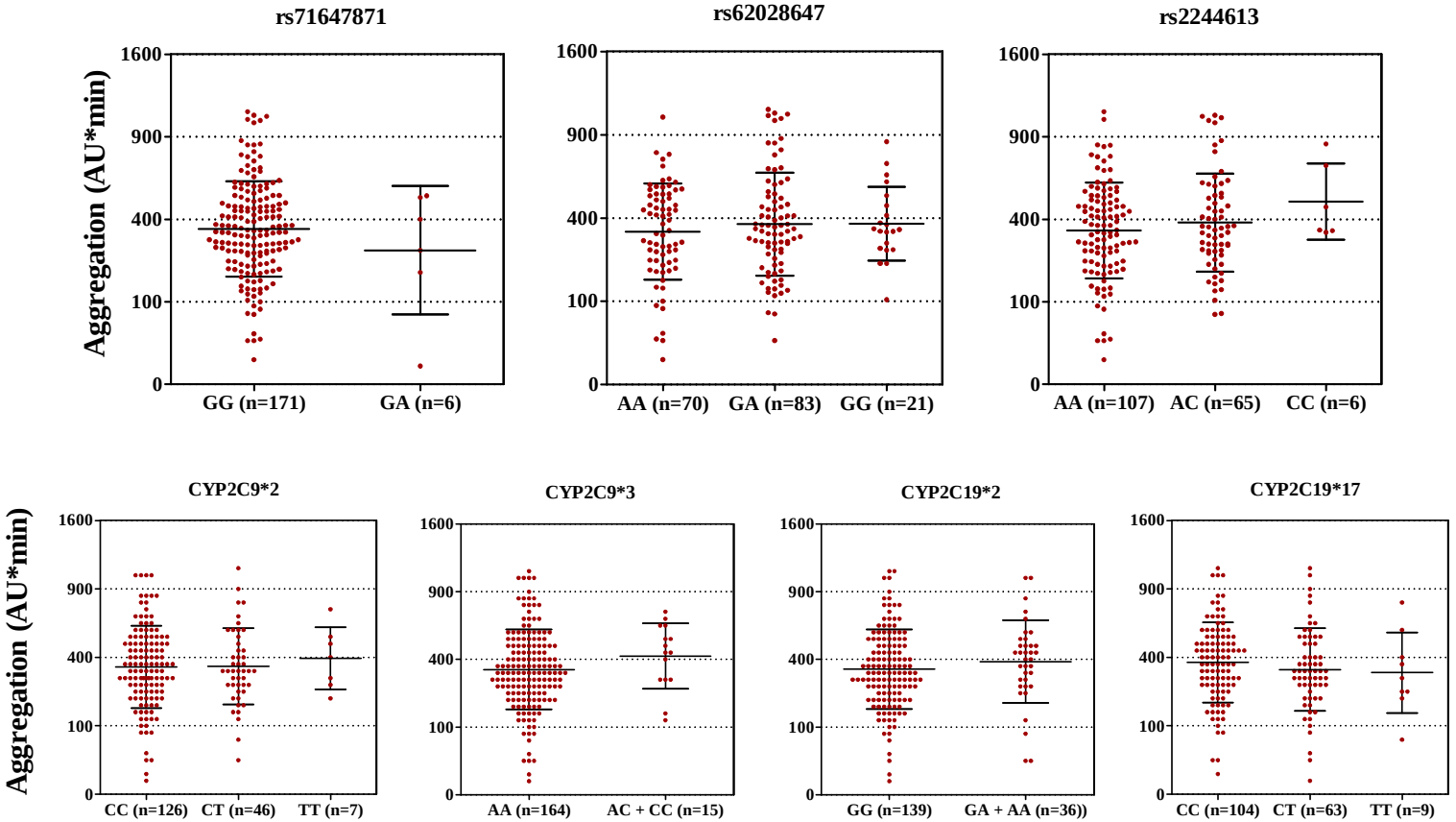


**Figure 3-5 Impact of CES1, CYP2C9 and CYP2C19 SNPs genotype on CLP-induced platelet aggregation.** The lines and bars indicate mean and SD, n=subject number. Y axis is displayed in a back transformed SQRT-scale.

**Table 3-4 Univariate and multivariate analysis of the effect of demographic, clinical and genetic variables on platelet aggregation in ACS patients treated with clopidogrel.**

| Variables | Univariate | Multivariate |
|---|---|---|
| **Age** | 0.981 | - |
| **Gender** | 0.742 | - |
| **Ethnicity** | 0.203 | - |
| **Diabetes** | 0.696 | - |
| **Smoking status** | **0.100** | - |
| **rs71647871** | 0.289 | 0.236 |
| **rs62028647** | 0.735 | 0.778 |
| **rs2244613** | **0.188** | **0.034** |
| **CYP2C9*2** | 0.783 | 0.741 |
| **CYP2C9*3** | 0.247 | 0.519 |
| **CYP2C19*2** | 0.283 | 0.374 |
| **CYP2C19*17** | 0.235 | 0.18 |

### 3.3.4. Demographics and genetic variants impact on CLPM plasma concentration

CLPM quantification was performed on 46 plasma samples from patients for which aggregation data had been measured at visit 2 (1 month) by the Multiplate® analyser. 5 of the samples returned concentrations under the LOD and therefore were excluded from the analysis. The remaining 41 samples were assessed for normality by Shapiro-Wilk test, as well as observation of histograms and Q-Q plots. Transformation by square root was performed in the same way as described for the platelet aggregation data.

The impact of age, sex, diabetes and smoking status on CLPM plasma concentrations is depicted in Figure 3-6. The impact of CES1, CYP2C9 and CYP2C19 SNPs on CLPM plasma concentrations is depicted in Figure 3-7. Genotypes carried by 5 or less individuals were grouped together with heterozygous genotypes in the figures and the analysis.

Univariate analysis of variance was performed to identify significant differences between means, only sex arose as a significant predictor of CLPM plasma levels (P=0.002). A multifactorial analysis was performed by general linear model including the variables with a P value of less than 0.200 meaning sex was selected as a correcting factor. No SNP was found significantly correlated with CLPM plasma concentrations after correcting. There was no significant correlation between platelet aggregation and CLPM plasma levels. The results of both univariate and multivariate analysis can be found in Table 3-5.

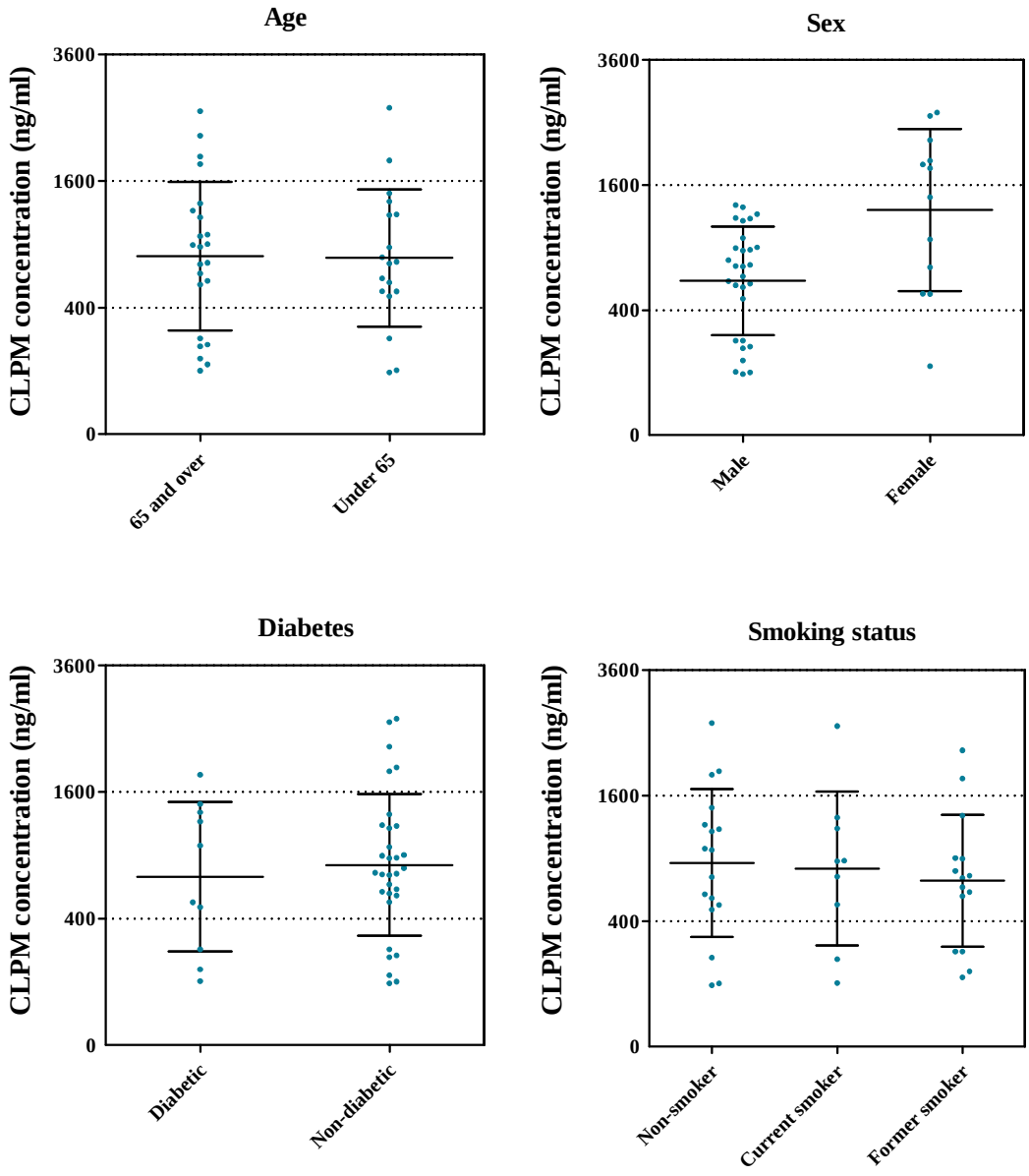


**Figure 3-6 Impact of demographic and clinical factors on CLPM plasma concentration.** Scatter dot plots of CLPM plasma concentration data by age, sex, diabetes and smoking status. The lines and bars represent the mean and the SD. Y axis is displayed in a back transformed SQRT-scale.

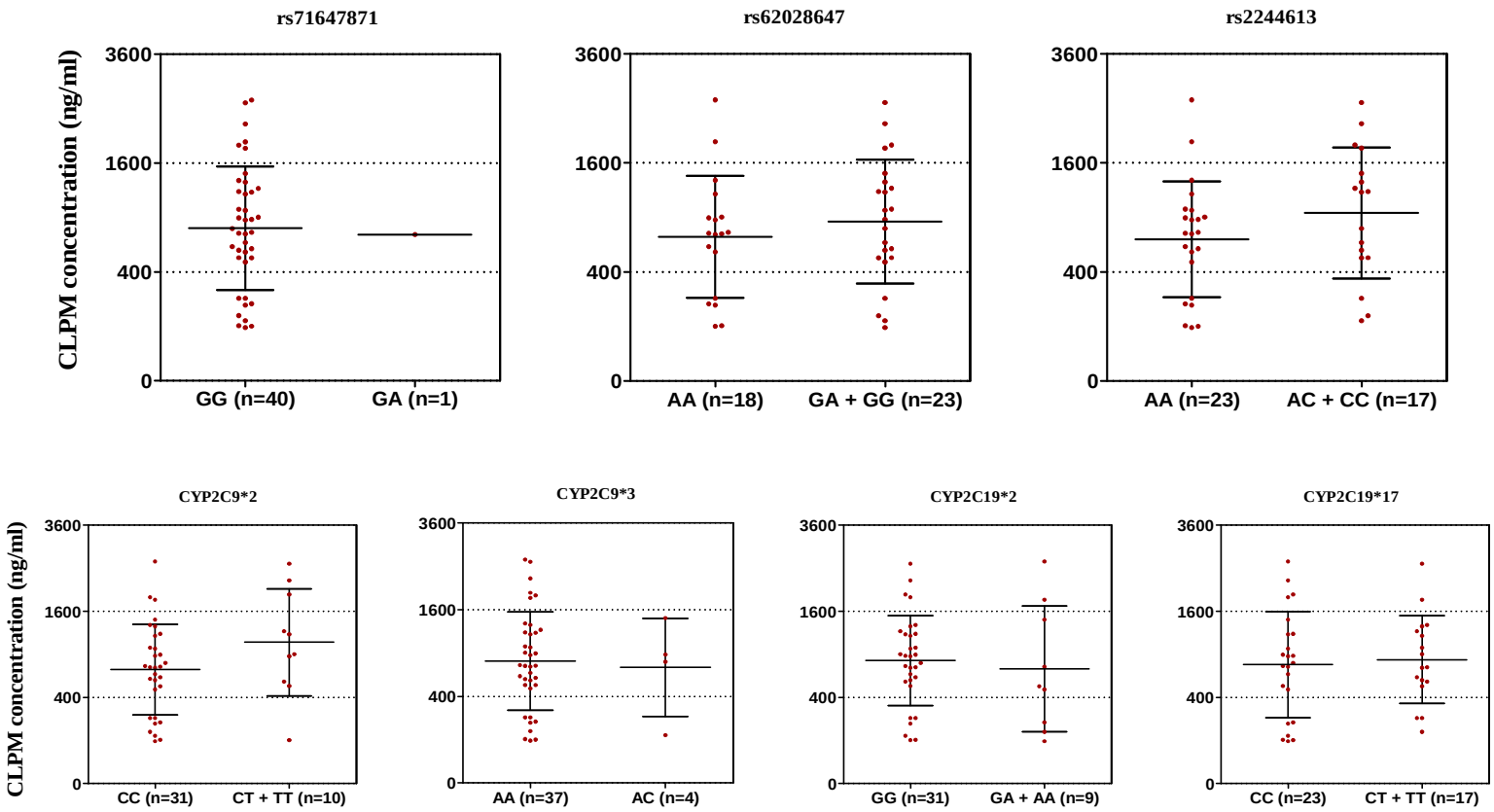


**Figure 3-7 Impact of CES1, CYP2C9 and CYP2C19 genes SNPs genotype on CLPM plasma concentration**. Scatter dot plots of CLPM plasma concentration data by genotype. The lines and bars represent the mean and the standard deviation (SD), n = subject number. Y axis is displayed in a back transformed SQRT-scale.

**Table 3-5 Univariate and multivariate analysis of the effect of demographic, clinical and genetic variables on CLPM plasma concentrations in ACS patients treated with clopidogrel.**

| Variables | Univariate | Multivariate |
|---|---|---|
| **Age** | 0.941 | - |
| **Gender** | **0.002** | - |
| **Diabetes** | 0.656 | - |
| **Smoking status** | 0.779 | - |
| **rs71647871** | 0.919 | 0.348 |
| **rs62028647** | 0.591 | 0.339 |
| **rs2244613** | **0.184** | 0.810 |
| **CYP2C9*2** | **0.122** | 0.200 |
| **CYP2C9*3** | 0.806 | 0.432 |
| **CYP2C19*2** | 0.665 | 0.287 |
| **CYP2C19*17** | 0.750 | 0.461 |

### 3.4. Discussion

The work developed in this chapter attempted to improve our knowledge of how genetic variability in the human CES1 gene affects response to antiaggregant therapy with the widely prescribed drug CLP. We found evidence that carriers of the minor allele of rs2244613 SNP (C) achieve significantly higher platelet aggregation values than WT (AA).

This work involved CES1 variants whose effect had been previously assessed by other groups or predicted by *in silico* tools such as Polyphen-2 and SIFT. The frequencies found in our population are in general similar to those previously reported in Caucasian populations except for rs3826190 that had a reported MAF of 0.30 but was here found monomorphic.

Linkage disequilibrium was explored between the SNPs included in the analysis using HaploView. Two CES1 SNPs appeared highly linked (rs2244613 and rs62028647), the rest of the CES1 SNPs could not be assessed because of low frequencies of minor alleles. The linkage found between the SNPs in CYP2C9 and CYP2C19 is not surprising as both genes sit on chromosome 10.

None of the demographic and clinical factors explored in this study exerted significant effects on platelet aggregation, although current and former smokers showed a trend towards lower platelet aggregation. This is consistent with what the literature calls the ‘smokers paradox’ where smokers obtain significantly greater benefit from CLP treatment than non-smokers i.e. they have lower risk of suffering major cardiovascular events (Williams 2014).

For the genetic assessment, SNPs were analysed independently and in multifactorial models. After accounting for smoking status individuals carrying a minor allele of rs22444613 scored significantly higher platelet aggregation values. There were no significant differences in mean platelet aggregation between genotypes of any of the other SNPs assessed. However, the overall means shown in the graphic display of the data go in the direction expected from our mechanistic understanding, and maybe significant associations could have been found in a larger cohort.

Sex significantly affected CLPM concentrations in this cohort, with females displaying significantly higher concentrations than men. This is consistent with the literature where females have been reported to show higher CES1 catalytic efficiency than males (Patrick et al. 2007). There were no significant differences in CLPM concentration between genotypes of any of the SNPs assessed in this chapter.

Moreover CLPM concentration data should be considered carefully given the likely variability in dosing to sampling time for these samples. This is evident in the spread of the data and may explain why we find a significant effect of rs2244613 with platelet aggregation but not CLPM concentrations.

Rs2244613 had been found significantly associated with reduced trough concentrations of the anticoagulant dabigatran and related bleeding, in the first ever study to report genome wide significance for a CES1 variant (Pare et al. 2013). It also showed a significant association with the occurrence of the adverse effect of sadness in children treated with methylphenidate (Johnson et al. 2013).

Both drugs mentioned are CES1 substrates, which suggests a functional effect of this intronic variant on enzyme expression or activity but the exact functional mechanism has not yet been unravelled. Our work indicates that rs2244613 may be a high activity variant of the gene.

rs2244613 appeared in our cohort in high linkage disequilibrium with rs62028647, which is a non-synonymous mutation, but these results do not support the idea that rs62028647 is the functional allele of the haplotype as it does not seem to exert any major effect on either platelet aggregation or CLPM levels. Further research is necessary to clarify if the effects found associated with rs2244613 are functional or due to another linked SNP.

Rs71647871 (Gly143Glu) is a naturally occurring genetic variant that has been reported to have a rather strong effect on CES1 activity (Zhu et al. 2008). Despite its low frequency (2-5%) significant effects on CLP pharmacokinetics and activity have been reported (Lewis et al. 2013, Zhu et al. 2013). In this chapter, no significant association was found with CLPM concentration in plasma or platelet aggregation, which could be due to insufficient sample size, since only 6 variant allele carriers were found in a cohort of 187 subjects.

Regarding CYP2C19, even though not significant, both the low activity variant (CYP2C19*2) and the high activity variant (CYP2C19*17) follow the expected trend in platelet aggregation. Evidence about CYP2C19 genotype importance on CLP treatment has been conflicting but the association between loss-of-function CYP2C19 alleles and risk of cardiovascular events was confirmed in meta-analysis (Hulot et al. 2010).

The currently accepted gold standard method to measure platelet aggregation in the clinical setting is Light Transmission Aggregometry (LTA). In this study an alternative method was used, the Multiplate® system. This allowed us to measure the antiplatelet effect due exclusively to CLP, which was our target drug. Moreover, it was advantageous considering this study was observational and many of the patients could be concomitantly treated with more than one anticoagulant agent. This, however, does not make up for the amount of confounding factors that reduce the power of our results, including co-medications, genetic variability on other enzymes, transporters or platelet ADP receptors.

In addition, measuring CLPM may be advantageous to assess CES1 variability as it is the direct product of the enzyme, but it is not the best marker for CYP P450 activity or to correlate with platelet aggregation, for which a measure of the active metabolite would be more suitable. However, measurement of both the active metabolite and the parent compound was not achievable with the HPLC/UV methodology used for this determination. More sensitive technologies, such as LC/MS-MS, can be used to determine their concentrations. Such technology is however not always readily available in all laboratories and the cost of purchasing and maintaining them is significantly greater than an HPLC/UV instrument.

In conclusion, further assessment of CES1 variants in larger populations is necessary to clarify the impact of CES1 variability on CLP metabolism and activity. There is a growing body of evidence being gathered by groups around the world suggesting that genetic variability of human CES1 enzyme may play a bigger role in drug metabolism than that previously suspected.

# CHAPTER 4

# Effect of efavirenz and nevirapine on the disposition of antiplatelet agent clopidogrel in HIV positive subjects

# Chapter 4: Effect of efavirenz and nevirapine on the disposition of antiplatelet agent clopidogrel in HIV positive subjects

## 4.1. Introduction

Human immunodeficiency virus (HIV) affects an estimated 35 million people worldwide. Due to improvements in antiretroviral (ARV) treatments, adherence and diagnosis, life expectancy of HIV positive individuals is approaching that of the general population (Lewden et al. 2012). In fact, approximately 3.6 million individuals affected by HIV are aged 50 years or above (UNAIDS 2013).

Unfortunately, this also translates into an increased risk of drug-drug interactions (DDIs) between antiretroviral therapy (ART) and treatments for chronic diseases and other aetiologies associated with old age, such as diabetes, cancer and cardiovascular disease.

Furthermore, HIV infected individuals are more likely to develop cardiovascular pathologies because of inflammatory processes derived from the disease progression (Stanley and Grinspoon 2012) as well as a consequence of treatment. In fact ART with abacavir and HIV protease inhibitors (PIs) has been linked to an increased risk of suffering myocardial infarction (Bavinger et al. 2013).

Highly active antiretroviral therapy (HAART) for HIV usually consists of the administration of three or more ARV drugs in a combination that includes PIs, nucleoside analogues (NA), integrase inhibitors (InIs), entry inhibitors (EIs) and non-nucleoside reverse transcriptase inhibitors (NNRTIs).

NNRTIs, such as efavirenz (EFV) and nevirapine (NVP), interact with HIV reverse transcriptase enzyme in a non-competitive way, thus interfering with viral replication. Both compounds undergo oxidation by cytochrome P450 enzymes (CYP P450). In particular, EFV metabolism is heavily mediated by CYP2B6 and to a lesser extent by CYP2A6 and CYP3A4/5 (Ward et al. 2003). NVP is also metabolized by CYP2B6 and CYP3A4 (Wen et al. 2009).

EFV and NVP are known to induce their own metabolism as well as that of a number of other pharmacologically active compounds, in a mechanism believed to be via induction of the pregnane X receptor (PXR) and constitutive androstane receptor (CAR), two nuclear receptors heavily involved in exogenous metabolism.

Long-term exposure to EFV affects CYP2B6 but also a number of other enzymes whose expression is also governed by PXR and CAR, such as CYP3A4 and CYP2C19 (Ngaimisi et al. 2010, Michaud et al. 2012).

Interestingly, EFV has been shown to exert short-term competitive inhibition on CYP2C19 *in vitro* (von Moltke et al. 2001). Michaud et al. (2012) later confirmed this short-term inhibition *in vivo* as well as reporting an induction on CYP2C19 in the long-term.

As explained in Chapter 3, CYP2C19 is currently regarded as the main CYP P450 enzyme in the activation cascade of the antiplatelet drug clopidogrel (CLP). Therefore, a long-term induction of CYP2C19 by NNRTIs could theoretically impact CLP metabolism and exposure. Conversely, CLP is a potent CYP2B6 inhibitor (Richter et al. 2004).

With the rise in life expectancy among HIV positive individuals as well as the increased propensity of this population to develop cardiovascular conditions it would be expected to find a significant number of individuals co-treated with NNRTIs and CLP. A study was conducted in a Korean population to assess the effect of CLP on EFV metabolic ratios where it was shown that EFV metabolism shifted from the CYP2B6-mediated pathway, that yielded 8-hydroxyefavirenz, to a CYP2A6-mediated pathway with 7-hydroxyefavirenz as the product (Jiang et al. 2013).

However, the effect of NNRTIs on CLP metabolism has not yet been assessed. The aim of the work described in this chapter was to generate evidence on the effect that the co-administration of the NNRTIs, NVP and EFV, may have on CLP pharmacokinetics (PK). In particular, an interaction via carboxylesterase 1 (CES1) was explored since CES1 catalyses what is quantitatively the main metabolic reaction of CLP: the hydrolysis of CLP into its carboxylic metabolite (CLPM). To do so, CLPM levels were measured and used as an indicator of CLP PK. As explained in Chapter 2, measurement of CLP parent and active metabolite concentrations are too low to be quantified by our HPLC/UV technique.

## 4.2. Methods

### 4.2.1. Study design

The study cohort came from a phase IV, open label, sequential, randomized, proof-of-concept, multi-dose study led by the Department of Molecular and Clinical Pharmacology, University of Liverpool. HIV positive subjects were recruited between March 2010 and January 2012 at the Royal Liverpool University Hospital and Manchester Royal Infirmary, United Kingdom.

Enrolled individuals had a screening visit prior to study commencement in order to report basic demographic information and current and past medical history; carry out a physical examination and electrocardiogram (ECG); and provide blood for determination of physiological and biochemical parameters, such as liver function, full blood count including CD4 count and viral load.

Study subjects were instructed to take 75 mg of CLP (Plavix®, Sanofi Synthelabo, Guilford, United Kingdom) orally once daily for 7 days as well as continuing their previous NNRTI-containing ARV regimen. NVP dose was 200 mg 12 hourly and EFV dose was 600 mg daily. Blood was collected on day 8 at 0, 1, 2, 4, 8 and 12 hours and centrifuged to obtain plasma for PK analysis. Treatment compliance was determined by self-report.

An earlier phase of the study, not described here, measured EFV and NVP plasma levels in the absence and presence of CLP and evaluated the effect of CLP on EFV and NVP disposition. It must be noted that subjects were administered a standard breakfast before drug dosing and must be considered to be in a fed state. Details about the study design can be found in Figure 4-1.

The inclusion criteria of the study included being HIV positive with a viral load below 40 copies/ml, being 18 years of age or above, having been on treatment with NNRTIs for at least 6 months and not having shown signs of toxicity or allergy towards NNRTIs or thienopyridines.

The demographic exclusion criteria of the study were poor adherence to ART, pregnancy and intra-venous drug usage. Clinical exclusion criteria included past medical history of inter-current acute illness, coronary heart disease, bleeding diathesis, a platelet count below 100 x $10^9$/L, and a neutrophil count below 1.0 x $10^9$/ml.

Therapeutic compounds known to modify expression or activity of CYP2B6 were not allowed during the duration of the study. Among them, CYP2B6 inducers like carbamazepine and phenytoin or CYP2B6 inhibitors such as amiodarone and HIV PIs.

The study protocol was approved by the North West research ethics committee in August 2009. Written informed consent was signed by all participants during the screening visit.

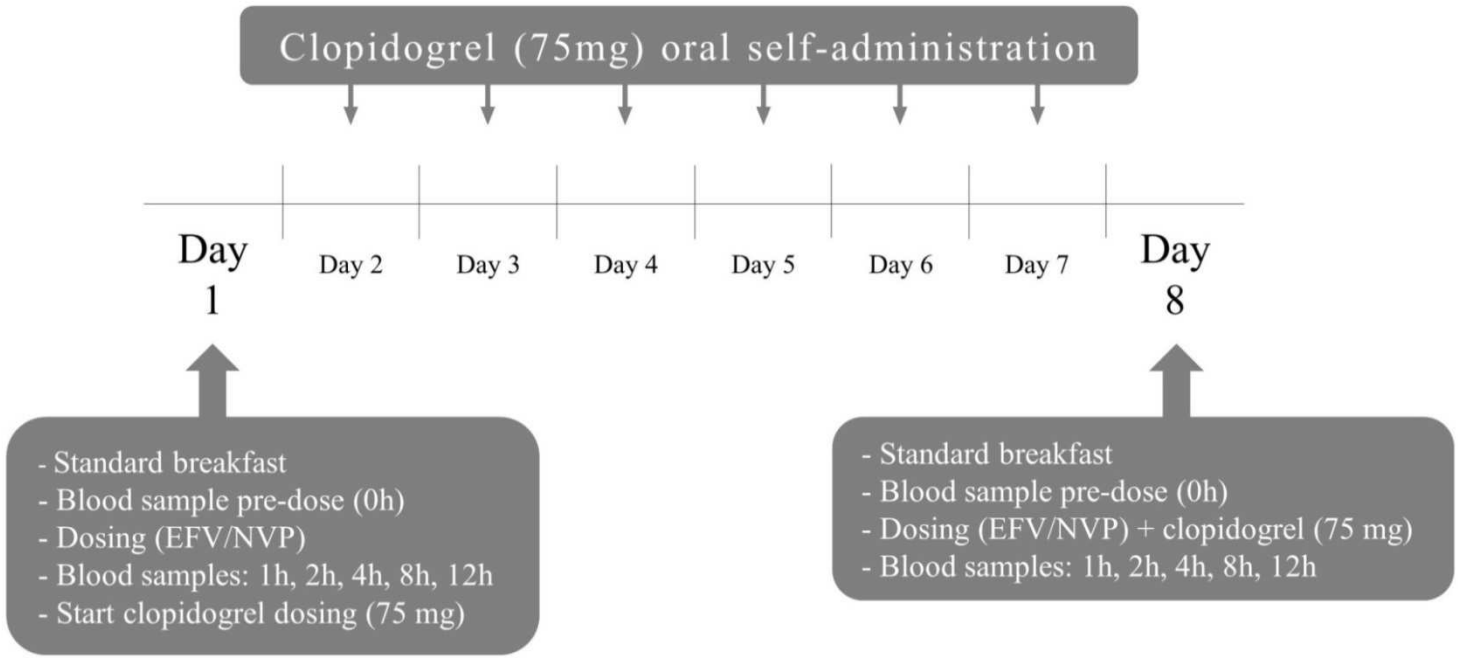


**Figure 4-1 CLP-NNRTIs interaction study design:** Study subjects were instructed to take 75 mg of CLP orally once daily for 7 days. Blood was collected on days 1 and 8 at 0, 1, 2, 4, 8 and 12 hours for PK analysis.

### 4.2.2. Determination of CLPM plasma concentration

Development and validation of the methodology used to quantify CLPM has been explained in detail in section 2.2. Briefly, measurement was carried out with a High Performance Liquid Chromatography technique with ultraviolet detection (HPLC/UV) instrument at a detection wavelength of 200 nm. The column employed was a Hypersyl gold 150x4.6 mm and 5 µm of particle size (Thermo Scientific Inc. Waltham, Massachusetts, USA). A gradient was applied with varying concentrations of organic solvent (acetonitrile:water, 90:10) and aqueous solvent ($KH_2PO_4$ 10 mM with 200 µl orthophosphoric acid (OFA), pH =3.2). Flow rate was 1 ml/min. Liquid-liquid extraction was performed with ethyl acetate on 200 µL volume of plasma. The limit of detection (LOD) was 78 ng/ml. Quantitation was linear for plasma concentrations between 78 and 10,000 ng/ml.

### 4.2.3. PK and statistical analysis

Metabolite maximal concentration ($C_{max}$), time at maximal concentration ($t_{max}$), elimination half-life ($t_{1/2}$) and area under the concentration-time curve (AUC) both at the last recorded time point ($AUC_{0-t}$) and extrapolated to infinity ($AUC_{0-\infty}$), were calculated for CLPM using standard non-compartmental methods. AUC was calculated by the trapezoidal calculation method.

Data were plotted and analysed using Graphpad Prism v. 5.01 (La Jolla, California, USA). Normality was assessed using the Shapiro-Wilk test. Comparison of parameters was carried out by unpaired t-test for normally distributed data. P values to an alpha of less than 0.05 were considered statistically significant.

In the absence of a CLP-alone control phase in our study, historical data obtained from studies that also quantified CLPM after 75 mg daily dose of CLP were used as a reference for PK parameters comparison (Karazniewicz-Lada et al. 2012, Brvar et al. 2014). CLPM PK parameters were calculated for the whole cohort (n=9) and also for NVP (n=4) and EFV (n=5) treated groups, separately.

## 4.3. Results

### 4.3.1. Subject characteristics

A total of 10 individuals were screened for the study and 9 were ultimately enrolled. All subjects were HIV positive, were receiving ART with a NNRTI at the time of enrolment and had been on the regimen for at least 6 months prior to the study commencement. 4 of the subjects were on treatment with NVP and 5 were treated with EFV. Subjects were dosed with 75 mg of CLP once daily for a week and blood was collected on day 8 at 0, 1, 2, 4, 8 and 12 hours after the last CLP administration. The demographic information and concomitant medications of the subjects enrolled can be found on Table 4-1. Low enrolment was the reason why recruitment for the study was stopped.

**Table 4-1 Demographic and pharmacological data of study participants Information on demographics and concomitant treatments of study participants are shown as number of individuals (n) and percentage of the population they represent (%). Age, weight and height are shown as mean with standard deviation (SD) and range.**

| | | |
|---|---|---|
| **Demographics** | | |
| Sex | Male (n, %) | 6 (66.7) |
| | Female (n, %) | 3 (33.3) |
| Age (y) | Mean (SD) | 42.8 (7.2) |
| | Range | 29-53 |
| Weight (kg) | Mean (SD) | 80.6 (12.4) |
| | Range | 60.0-93.1 |
| Height (m) | Mean (SD) | 1.73 (0.09) |
| | Range | 1.62-1.87 |
| Ethnicity | Black African (n, %) | 5 (55.6) |
| | White English (n, %) | 4 (44.4) |
| **Pharmacology** | | |
| NNRTI drug | Nevirapine (n, %) | 4 (44.4) |
| | Efavirenz (n, %) | 5 (55.6) |
| Other HIV drugs | Abacavir (n, %) | 3 (33.3) |
| | Lamivudine (n, %) | 3 (33.3) |
| | Tenofovir (n, %) | 2 (22.2) |
| | Lopinavir (n, %) | 1 (11.1) |
| | Ritonavir (n, %) | 1 (11.1) |
| | Emtricitabine (n, %) | 1 (11.1) |
| Other treatments | Other antiinfectious | 3 (33.3) |
| | Antihypertensive | 2 (22.2) |
| | Supplements and vitamins | 2 (22.2) |
| | Other | 3 (33.3) |

### 4.3.2. PK assessment and comparison to historical data

$C_{max}$, $AUC_{0-t}$ , $AUC_{0-\infty}$ , $T_{max}$ and half-life parameters calculated for the full cohort can be found in Table 4-2.

Normality of PK parameters was evaluated using the Shapiro-Wilk test. There were no deviations from a Gaussian distribution.

Historical data obtained from previously published studies that quantified CLPM in subjects treated with a daily dose of 75 mg of Plavix® (Karazniewicz-Lada et al. 2012, Brvar et al. 2014) can also be found in Table 4-2.

The current study cohort displays generally lower $C_{max}$, while $AUC_{0-t}$. $AUC_{0-\infty}$ and half-life do not appear markedly different. As expected, CLPM PK behaviour in the current study appears more similar to the fed leg of the Brvar et al. study.

Concentration-time curves obtained from all study subjects are depicted in Figure 4-2a on a logarithmic scale. They all followed a similar pattern except for subject 7 (in green) which exhibited an unusually low exposure at early time points; the possible reasons for this are discussed in section 0

**Table 4-2 CLPM PK data and study characteristics from previously published studies and the current cohort. Mean values of PK parameters for subjects treated with NVP and EFV separately are also shown. P values of the difference between PK parameters in NVP and EFV subgroups were obtained by unpaired t-test analysis and are shown in the last column. Data are displayed as the mean and standard deviation (SD).**

| | Historical data | | | Current study | | | |
|---|---|---|---|---|---|---|---|
| | **Karazniewicz-Lada et al. 2012** | **Brvar et al. 2014** | **Brvar et al. 2014 (2)** | **Full cohort** | **NVP group** | **EFV group** | **P-value** |
| **Dose** | 75 mg | 75 mg | 75 mg | **75 mg** | 75 mg | 75 mg | - |
| **N** | 3 | 92 | 113 | **9** | 4 | 5 | - |
| **$C_{max}$ (ng/ml)** | 2821 (519) | 2609 (855) | 2194 (655) | **1309 (553)** | 1320 (482) | 1300 (661) | 0.9627 |
| **AUC $_{0-t}$ (ng/ml*h)** | 9782 (6518) | 6775 (1436) | 6510 (1195) | **5418 (2077)** | 4720 (1422) | 5976 (2496) | 0.4028 |
| **AUC 0-∞ (ng/ml*h)** | 10945 (7075) | 7230 (1536) | 6836 (1267) | **7803 (3135)** | 5751 (1919) | 9444 (3053) | 0.0744 |
| **$T_{max}$ (h)** | 1.7 (0.6) | 0.8 | 1.5 | **1.3 (0.4)** | 1.2 (0.3) | 1.3 (0.5) | 0.7768 |
| **Half life (h)** | 4.0 (1.3) | 8.9 (2.1) | 7.4 (1.5) | **6.4 (3.9)** | 4.1 (1.3) | 8.2 (4.4) | 0.1216 |
| **Fed state** | Fast | Fast | Fed | **Fed** | Fed | Fed | - |
| **Subjects** | ACS patients | Healthy volunteers | Healthy volunteers | **HIV infected** | HIV infected | HIV infected | - |

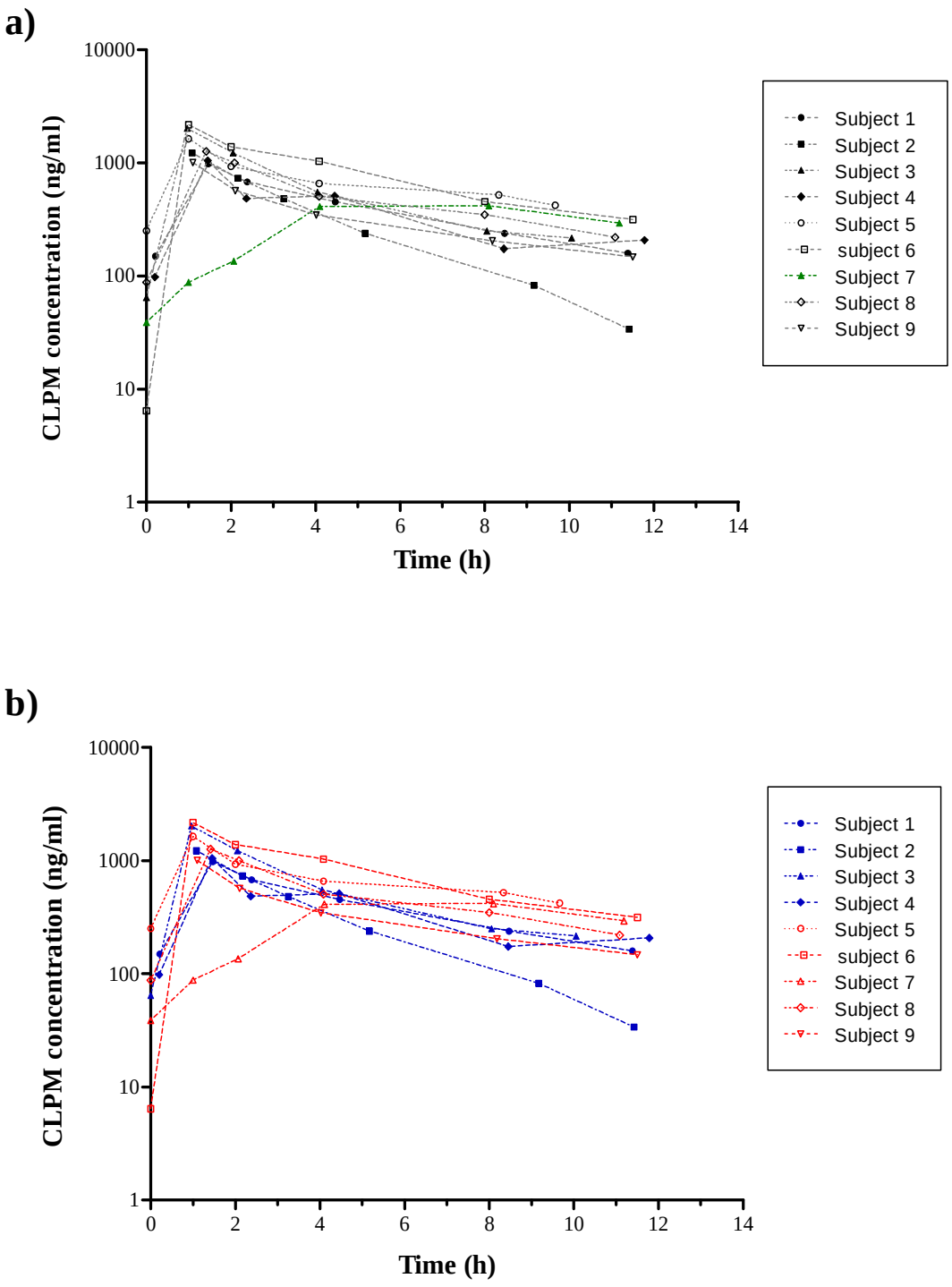


**Figure 4-2 CLPM concentration-time curves.** a) CLPM study subjects individual plasma concentration-time profiles following one week co-administration of CLP with NNRTIs, CLP dose was 75 mg once daily. b) Subjects split in subgroup treated with NVP (blue) and subgroup treated with EFV (red).

### 4.3.3. Differences between NVP and EFV treated subjects

Data from the current study subjects were split between those co-treated with NVP and those with EFV. CLPM concentration-time curves are shown in Figure 4-2b. In this figure it is apparent that, even though $C_{max}$ do not appear markedly different between groups, EFV treated subjects display lower rates of elimination towards the end.

PK parameters were calculated for each of the treatment groups and compared by unpaired t-test analysis; the results are shown in Table 4-2. $C_{max}$ and $T_{max}$ did not appear different; however, $AUC_{0-t}$ and, especially, $AUC_{0-\infty}$ were higher in EFV treated subjects. Half-life in the EFV group (8.2 h) was also longer than in the NVP group (4.2 h). Neither difference was statistically significant which may be due to the low n-numbers in the separate groups and the study overall.

## 4.4. Discussion

Therapies for HIV and cardiovascular disease are increasingly administered together and there is a need to identify and characterize the interactions between these treatments that may affect their efficacy and safety. In this small study an interaction between NNRTIs and CLP was explored. The results show that some potential differential patterns between NVP and EFV treated subjects in CLP PK may occur.

NNRTIs and CLP are drugs which could affect each other's metabolism by modifying expression and activity of the enzymes involved in their disposition. Work had been developed to characterize the impact of CLP on EFV disposition via CYP2B6 (Jiang et al. 2013), but the influence of NNRTIs on CLP metabolism has not yet been assessed. The work developed in this chapter explores putative interactions between NNRTIs and CLP, focusing on CES1 as the mediator.

Results were compared to historical data in the absence of a CLP-alone control group and the overall results did not appear markedly different to those observed in similar previously published studies, except maybe for a lower $C_{max}$. Therefore the data available from this study does not support the idea that NNRTIs affect CLP PK. This may imply that the concurrent administration would not impair CLP antiplatelet efficacy.

However, when splitting the study cohort into those receiving NVP and those receiving EFV and comparing their PK parameters, subject differences between $AUC_{0-\infty}$ and predicted half-lives appeared to be shown.

Statistical analyses were carried out to compare mean values of PK parameters but due to the small sample size, no statistically significant difference was identified. Therefore it cannot be assured that the observed differences are not due to chance.

It must be emphasized that CLPM, the metabolite quantified in this study is quantitatively the main CLP metabolite and the direct product of the hydrolysis by CES1, allowing a more direct observation of interactions mediated by this enzyme. As $C_{max}$ remains similar between the two groups, the differences in $AUC_{0-\infty}$ and half-life may be due to a difference in its elimination, perhaps by competition for a transporter between metabolites.

EFV inductive effects on CYP2C19 and CYP3A4 have previously been observed (Michaud et al. 2012). Both these enzymes are part of the CLP activation cascade (See Figure 3-1). An increase in CYP P450 activity could theoretically compete with CES1 for CLP parent compound and increase the fraction of the absorbed dose that enters the activation pathway. In such a case, we would expect to observe a decrease in CLPM exposure derived from a smaller amount of CLP entering the CES1 pathway, which we do not observe even though subjects in this study had been treated for at least 6 months with an NNRTI before study commencement, enough time to develop maximal CYP P450 induction.

NNRTIs inductive effects are mediated by PXR and CAR (Michaud et al. 2012), and if CES1 were regulated by a similar intracellular pathway, changes in CLPM PK profile might have been apparent due to changes in CES1 expression. Since no such differences are observed between our study participants and CLP-alone volunteers, such premise is not supported. A further exploration of CES1 regulation of expression was conducted as part of this thesis, results are shown in Chapter 6.

Unfortunately, the small sample size of this study makes it difficult to make firm conclusions or assess the effects of factors such as cohort characteristics, dosage of drugs or fed/fasted state of subjects. Also, although comparison of PK parameters against previously published data can be useful, it would have been preferable to have a control group for CLP alone within the study to minimise the potential for systematic random differences in the data.

Other limitations of this study include the mixed ethnicity of the study cohort so variability derived from ethnicity cannot be identified since some of the variability in drug processing derives from single nucleotide polymorphisms (SNPs) frequency differences between ethnic groups. There are some genetic variants in CYP2C19 and CYP2B6 genes that have been shown to exert a considerable effect on drug processing (Rotger et al. 2007, Shuldiner et al. 2009) and they should be taken into account when assessing enzyme activity.

Genetic variability could also have helped explain the anomalous metabolic profile of subject 7, but DDIs could also be the cause of this unusual curve. The medical history of this subject was reviewed and it revealed that they were treated concomitantly with felodipine, which has been found in *vitro* to be a strong CES1 inhibitor, causing a reduction of 84% in CES1 catalytic activity (Yanjiao et al. 2013). This inhibition may be the cause of the unusual PK curve for patient 7, but specific *in vivo* studies would be necessary to assess the importance of this interaction.

Future work to develop in this area should include well designed, randomized *in vivo* studies to properly assess the clinical relevance of the impact of NNRTIs on CLP disposition. The PK assessment should include CLP and all its metabolites from both activation and inactivation pathways and a genetic analysis of common SNPs on the main enzymes involved in CLP disposition.

A pharmacodynamic assessment of CLP antiaggregant activity, like the one performed in Chapter 3, would inform us of any potential clinical repercussion, e.g. increased thrombotic risk from higher aggregation in EFV treated patients.

In addition, *in vitro* studies could also be used to clarify short-term competition between NNRTIs and CLP, however, due to long-term treatments with ART and its metabolic inductive effects, the usefulness of such studies to inform clinical practice would be limited.

In conclusion, this study represents the first observation of CLP PK in an HIV positive population treated with NNRTIs. Although not conclusive, due to small recruitment numbers, our data indicates that a reduced antiaggregant efficacy of CLP would not result from a concomitant administration with NNRTIs. The observed differences in CLPM disposition between NVP and EFV treated subjects cannot be confirmed nor refuted with the available data, but represent an interesting avenue for further study that should be explored before completely ruling out the possibility of a clinically relevant DDI.

# CHAPTER 5

# Impact of CES1 single nucleotide polymorphisms on isoniazid pharmacokinetics

# Chapter 5: Impact of CES1 single nucleotide polymorphisms on isoniazid pharmacokinetics

## 5.1. Introduction

It is estimated that one third of the world population carries *Mycobacterium tuberculosis*, the agent responsible for tuberculosis infection (TB). However, the majority of the population experiences a latent infection while only about 10% of individuals develop symptoms. Unfortunately, in individuals with a compromised immune system the risk of progression from latent to active infection is 21 to 34 times higher than in immunocompetent subjects. Individuals co-infected with TB and human immunodeficiency virus (HIV) are especially vulnerable. In fact, TB is the single most common cause of death among HIV infected subjects, being responsible for about one fifth of the overall HIV mortality with 1.1 million out of the 8.6 million new TB cases reported in 2012 in people living with HIV.

As a result, globally TB/HIV co-infection is currently one of the biggest challenges that tuberculosis treatment faces, together with the rise in drug-resistant TB strains (WHO 2013).

Precisely to avoid development of drug resistance, as well as reducing the risk of transmission, TB treatment always involves multiple pharmacological agents with what has come to be known as first and second line anti-TB medications.

First line anti-TB drugs are isoniazid (INH), rifamycins, ethambutol (EMB) and pyrazinamide (PZA). The rifamycin family includes rifampicin (RIF), rifabutin (RFB) and rifapentine (RPT). Second line drugs include cycloserine, ethionamide, levofloxacin or amikacin. Courses of anti-TB treatments last for a minimum of 6 months.

Treatment of TB in HIV co-infected subjects is similar to those mono-infected with TB but it carries further complications related to drug-drug interactions (DDIs) between antiretroviral therapy (ART) and anti-TB agents, such as RIF. RIF can be replaced by RBT which causes fewer interaction problems but this is not always available (Chien et al. 2014). In any case, concurrent initiation of anti-TB and anti-HIV treatments should be avoided and ART should be delayed 2-8 weeks after starting anti-TB treatment to avoid increased adverse events and paradoxical reactions (Naidoo et al. 2013). TB-HIV co-infection is also associated with reduced TB diagnostic test efficacy as well as higher rates of TB recurrence and transmission (Harries et al. 2010).

INH has been used for the treatment of tuberculosis for over 50 years and is currently prescribed for both treatment of active tuberculosis and chemoprophylaxis in latent tuberculosis (Wood and Bekker 2014). The appearance of hepatotoxicity, following initiation of INH treatment must be monitored since it is potentially fatal and has an incidence of 1 to 36% (Yamada et al. 2010). Factors like alcohol consumption, HIV co-infection, advanced age and liver disease are known to increase the risk of INH-induced hepatotoxicity (Metushi et al. 2011).

INH undergoes hepatic clearance via acetylation by N-acetyltransferase 2 (NAT2) and oxidation with cytochrome P450 enzymes (CYP P450). Genetic variability in NAT2 has been found to be associated with INH pharmacokinetics (PK) and development of hepatotoxicity (Sotsuka et al. 2011). Moreover, it has been suggested that liver amidases may be responsible for the hydrolytic steps in INH metabolism (Sarich et al. 1999).

Based on that study, the ability to cleave amide bonds and their abundance in the liver, Yamada et al. (2010) suggested that carboxylesterases may potentially play a role in INH disposition and related hepatotoxicity. Therefore they sequenced carboxylesterase 1 (CES1), carboxylesterase 2 (CES2) and carboxylesterase 4 (CES4) to assess if genetic variability in these genes had any effect on the development of hepatotoxicity. Their results did not show any single nucleotide polymorphism (SNP) significantly correlated with hepatotoxicity but some trends were identified (Yamada et al. 2010).

The aim of this chapter was to follow that line of reasoning and assess the impact of CES1 genetic variants on INH pharmacokinetics (PK) in a Thai cohort after a single INH administration. The effect of other factors such as demographic variables, HIV and TB infection was also explored.

## 5.2. Methods

### 5.2.1. Study design

Participants for this observational study were recruited from the Bamrasnaradura and Central Chest Hospitals in Bangkok, Thailand. Ethical approval was obtained from the Ethics Committees at Liverpool School of Tropical Medicine, Siriraj Hospital and Bamrasnaradura Institute and Central Chest Hospital.

Subjects recruited were included in one of four groups attending to their HIV and TB infection status. Group 1 included HIV and TB co-infected subjects, group 2 included TB positive only subjects, group 3 consisted of healthy volunteers, and group 4 included HIV positive only subjects.

Enrolled individuals had a baseline questionnaire prior to study commencement in order to record demographic and biometric information. Tests were performed for determination of physiological and biochemical parameters.

On the day of the study, subjects were cannulated prior to pharmacological treatment to obtain baseline PK data. A single dose of a weight-adjusted combination of INH/RIF/EMB/PZA was administered orally and 4 ml of blood were obtained at 5 time points between 0 and 11 hours for PK analysis.

The inclusion criteria included being between 18 and 65 years of age and having results of serology for HIV infection. For TB infected subjects it was necessary to have a positive sputum smear for acid-fast bacilli, non-drug-resistant TB and not having been on TB treatment within five years.

The exclusion criteria were ongoing illicit drug misuse or methadone therapy, pregnancy or lactation, current or immediate indications for ART, clinically significant anaemia or haemoglobin below 8 g/dl, significant renal dysfunction (serum creatinine > 177µmol/L (2 mg/dl)), and significant hepatic dysfunction (Total bilirubin > 51 µmol (3 mg/dl), AST>200 i.u./L).

### 5.2.2. DNA extraction and SNP genotyping

Genomic DNA was extracted from study subjects whole blood using the QIAamp DNA Blood Maxi Kit (Qiagen Inc., California, USA) following manufacturer's protocol. DNA was quantified using the Eppendorf BioSpectrometer® (Eppendorf ltd., Hamburg, Germany). Purity was assessed using the $A_{260}/A_{280}$ ratio. Samples were normalized to a concentration of 20 ng/ml (Davies 2008).

Genotyping was performed for the same CES1 SNPs described in Chapter 3, the list can be found in Table 5-1. The PCR reaction was carried out in Opticon 2 and Chromo4 real-time thermal cyclers (Biorad, Hemel Hempstead, UK). Off-the-shelf TaqMan® SNP Genotyping assays (Applied Biosystems, Foster City, California, USA) were used for DNA genotyping. Tests were performed in 96-well plates. The reaction volume was 25 µl of which 12.5 µl was TaqMan® Universal PCR Master Mix (Applied Biosystems, Foster City, California, USA), 1.25 µl was a mixture of probes and primers specific for each SNP, and 9.25 µl were PCR grade water (Sigma-Aldrich, St Louis, Missouri, USA). 2 µl of normalized sample (20 ng/ µl) were added to each well just before the start of the PCR reaction. Cycle conditions were 95ºC for 15 minutes followed by 50 cycles of 95ºC for 15 seconds and 60ºC for 1 minute.

**Table 5-1 Genetic variants explored by SNP genotyping in 84 individuals**

| Accession number | Gene | Chr. | Effect | Nucleotide change |
|---|---|---|---|---|
| rs71647871 | CES1 | 16 | p.G143E | G/A |
| rs71647872 | CES1 | 16 | p.R260- | T/- |
| rs62028647 | CES1 | 16 | p.S83L | A/G |
| rs3826190 | CES1 | 16 | p.G18V | G/T |
| rs2244613 | CES1 | 16 | Intronic | C/A |
| rs3815583 | CES1 | 16 | UTR-5 region | T/G |

### 5.2.3. INH plasma quantitation and PK parameters calculation

Full details of the bioanalytical method used to quantify INH in plasma can be found in Davies (2008). Briefly, the separation was carried out at the Therapeutics and Toxicology Laboratory, Llandough Hospital, Cardiff, using a High Performance Liquid Chromatography technique with ultraviolet detection (HPLC/UV). The specimen volume required was 1 ml of plasma. Extraction of analyte and internal standard (IS) were performed by solid phase extraction with an ISOLUTE column (Kinesis Ltd, Bedfordshire, UK). Iproniazid was used as IS. The chromatographic separation was performed with a Spherisorb nitrile column (5µm, 250 x 4.5 mm) (Waters ltd., Hertfordshire, UK) at a flow rate of 2 ml/min in an isocratic run. The mobile phase was composed of 8 mM citric acid and acetonitrile (80:20, v/v). UV detection was performed at a wavelength of 266 nm. The method's quantification range was 200-12,800 ng/ml.

PK parameters for INH were calculated with WinNonLin Professional version 5.0 (Pharsight Corporation, Mountain View, California, USA). A standard non-compartmental method was used. AUC was calculated by the trapezoidal method (Davies 2008).

### 5.2.4. Statistical analysis

A Chi-squared test was performed to explore significant deviations from Hardy-Weinberg equilibrium of genetic variants.

Normality of PK parameters was assessed by Shapiro-Wilk test as well as by observation of histograms and quantile-quantile (Q-Q) plots.

As part of the statistical analysis we explored whether demographic, clinical and genetic factors had any influence on PK parameters obtained from the study subjects' INH concentration-time curves.

Firstly, it was assessed if the underlying patient characteristics (e.g. age, sex) and the factors defined by the original experimental design (e.g. infection status, dose) influenced the PK parameters.

Secondly, it was assessed if CES1 genetic variants included in the study influenced the PK parameters.

These analyses were carried out in a structured way using general linear models in IBM SPSS® for Windows®, version 22 (SPSS, Illinois, USA). A single parameter approach was used to determine which factors to include in multifactorial models. The multifactorial models contained both main effect factors and factor interactions and were tested with and without corrections for patient demographic and clinical factors. To satisfy test assumptions, all statistical analyses were performed on log transformed values of $AUC_{0\text{-last}}$, $C_{max}$, and $\lambda_z$.

Figures were produced using Graphpad Prism v. 5.01 (La Jolla, California, USA) and IBM SPSS® for Windows®, version 22 (SPSS, Illinois, USA).

## 5.3. Results

### 5.3.1. Subjects demographics

A total of 86 subjects were included in the INH PK evaluation, their demographic data is summarized in Table 5-2. The mean age of study participants was 33.9, 44.2% of the participants were female and 55.8% were male. The mean BMI in the population was 19.8.

**Table 5-2 Study cohort overall demographic data**

| Demographics | | |
|---|---|---|
| Sex (n, %) | Female | 38 (44.2) |
| | Male | 48 (55.8) |
| Age (years) | Average (SD) | 33.9 (9.7) |
| | Range | 18-60 |
| BMI ($kg/m^2$) | Average (SD) | 19.8 (3.0) |
| | Range | 14.1-27.3 |

### 5.3.2. Frequencies of SNPs

SNP genotyping was carried out on 84 of the 86 participants. There was no DNA available for the remaining 2 subjects. The mean calling rate was 97.6%. The genotyping data obtained from SNP analysis can be found in Table 5-3.

SNPs rs71647871, rs71647872 and rs2826190 were monomorphic in this cohort. SNPs rs62028647 and rs3815583 displayed significant deviations from Hardy-Weinberg equilibrium; the reasons for this are discussed in section 5.4. of this chapter. For the subsequent analyses both homozygous mutants and heterozygous were grouped together and referred to as minor allele carriers. For the purpose of consistency, rs2244613 minor allele homozygous carriers were grouped with heterozygous individuals too.

**Table 5-3 CES1 SNPs genotyping results. N: Number of samples. $A_1/A_2$: Main and minor alleles. MAF: Minor allele frequency. H-W: Hardy Weinberg equilibrium. P value was obtained by Chi-squared test.**

| Accession Number | N | Calling rate | Genotype count | | | Genotype Frequency | | | MAF | H-W P value |
|---|---|---|---|---|---|---|---|---|---|---|
| | | | $A_1/A_1$ | $A_1/A_2$ | $A_2/A_2$ | $A_1/A_1$ | $A_1/A_2$ | $A_2/A_2$ | | |
| rs71647871 | 77 | 92% | 77 | 0 | 0 | 1.00 | 0.00 | 0.00 | 0.00 | N/A |
| rs71647872 | 84 | 100% | 84 | 0 | 0 | 1.00 | 0.00 | 0.00 | 0.00 | N/A |
| rs62028647 | 84 | 100% | 50 | 34 | 0 | 0.60 | 0.40 | 0.00 | 0.20 | 0.02 |
| rs3826190 | 79 | 94% | 79 | 0 | 0 | 1.00 | 0.00 | 0.00 | 0.00 | N/A |
| rs2244613 | 84 | 100% | 37 | 40 | 7 | 0.44 | 0.48 | 0.08 | 0.32 | 0.40 |
| rs3815583 | 84 | 100% | 31 | 53 | 0 | 0.37 | 0.63 | 0.00 | 0.32 | <0.01 |

### 5.3.3. Demographics impact on INH PK parameters

The influence of patient demographics (sex, age, BMI), INH dose (absolute and adjusted for weight) and TB and HIV infection status on INH PK parameters was investigated using a general linear model approach by a single factor model to detect main effects. The results are summarized in Table 5-4.

The single factor models showed significant positive relationships between BMI and both $AUC_{0\text{-last}}$ and $C_{max}$. Females displayed significantly higher values of $C_{max}$ and significantly slower elimination rates (lower $\lambda_z$) than males. Elimination rates ($\lambda_z$) were also positively correlated with INH adjusted dose (mg/kg) i.e. a quicker elimination for higher relative doses. Regarding infection status, $AUC_{0\text{-last}}$ was significantly higher in patients infected with HIV but lower in patients infected with TB. Additionally, TB infection also significantly lowered values of $C_{max}$.

The impact of categorical variables, such as sex, HIV and TB infection status on PK parameters are displayed in Figure 5-1. The impact of the continuous variable BMI on PK parameters is displayed in Figure 5-2. Correlation was investigated and $AUC_{0\text{-last}}$ and $C_{max}$ were significantly correlated with BMI.

**Table 5-4 Effect of single factors on PK parameters. Significant values to an alpha of 0.5 are highlighted in red. Beta is effect size.**

| Variables | $AUC_{0\text{-last}}$ | | $C_{max}$ | | $\lambda_z$ | |
|---|---|---|---|---|---|---|
| | **Beta [95% CI]** | **P value** | **Beta [95% CI]** | **P value** | **Beta [95% CI]** | **P value** |
| Sex | -0.102 [-0.342/0.138] | 0.401 | -0.195 [-0.382/-0.009] | 0.041 | -0.224 [-0.427/-0.021] | 0.031 |
| Age | -0.006 [-0.018/0.006] | 0.339 | -0.002 [-0.012/0.007] | 0.621 | -0.002 [-0.013/0.008] | 0.67 |
| BMI | 0.054 [0.015/0.093] | 0.007 | 0.048 [0.018/0.079] | 0.002 | -0.001 [-0.036/0.035] | 0.973 |
| Dose | 0.002 [-0.001/0.005] | 0.149 | 0.001 [-0001/0.004] | 0.195 | 0.001 [-0.002/0.003] | 0.555 |
| mg/kg | -0.087 [-0.328/0.154] | 0.473 | -0.008 [-0.200/0.184] | 0.935 | 0.291 [0.091/0.491] | 0.005 |
| HIV | -0.285 [-0.517/-0.054] | 0.016 | -0.063 [-0.253/0.128] | 0.515 | 0.176 [-0.028/0.381] | 0.09 |
| TB | 0.280 [0.048/0.512] | 0.019 | 0.202 [0.017/0.388] | 0.033 | 0.054 [-0.153/0.262] | 0.603 |

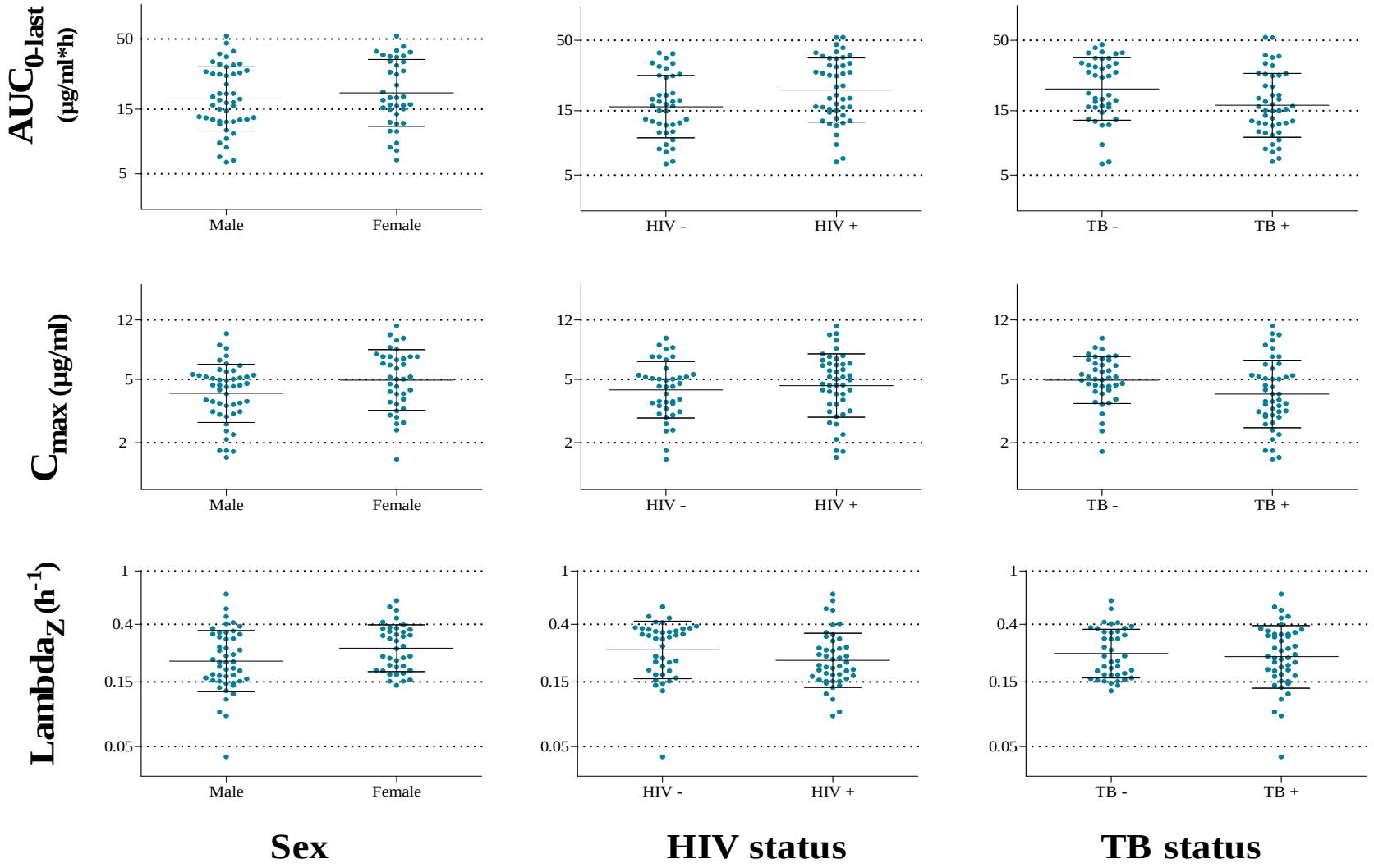


**Figure 5-1 Impact of categorical variables (sex, HIV and TB status) on PK parameters ($AUC_{0\text{-last}}$, $C_{max}$, and $\lambda_z$).** Scatter dot plots of PK parameters by sex, HIV status and TB status. The lines represent the mean and the standard deviation (SD). Y axis is displayed in a back transformed log-scale.

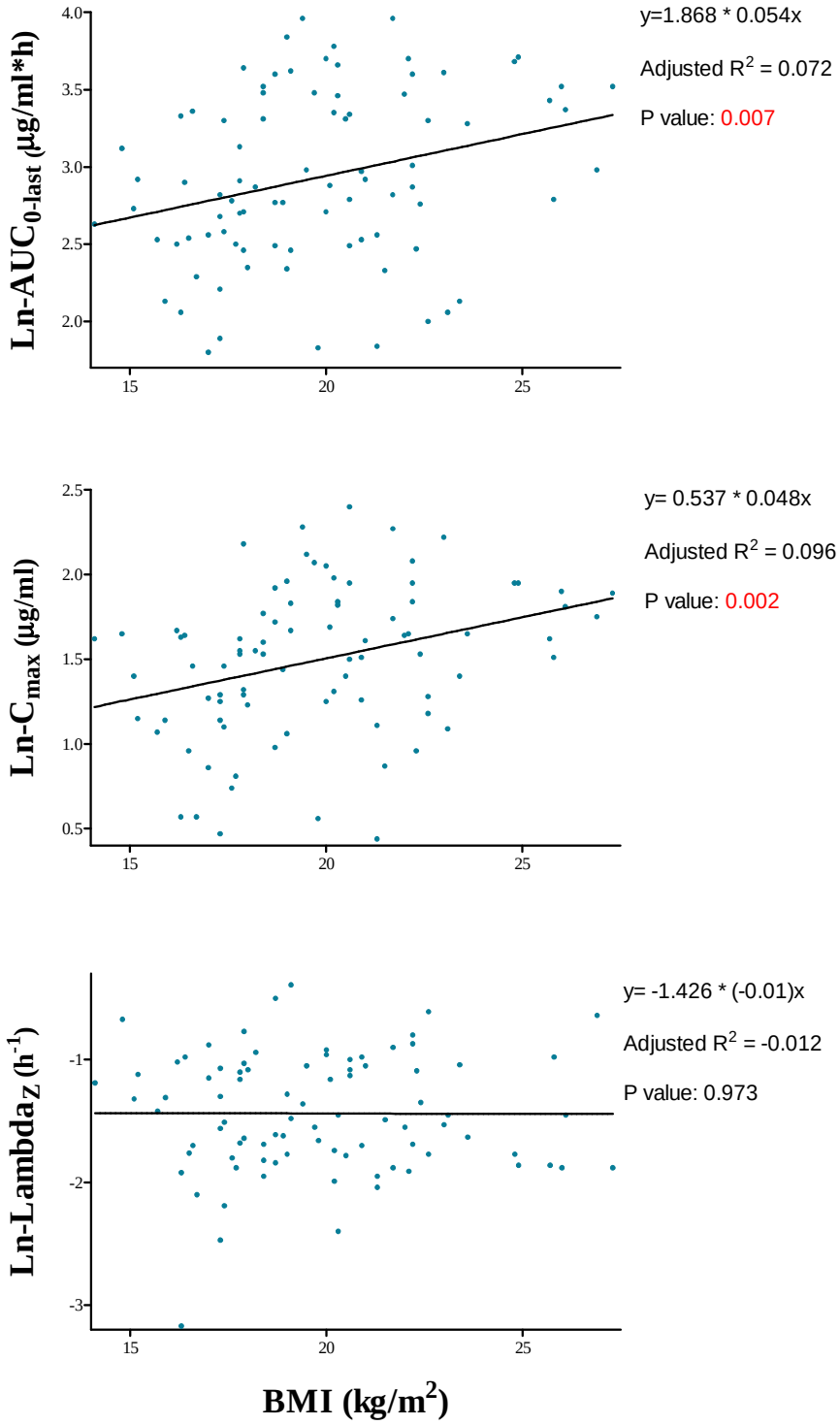


**Figure 5-2 Impact of BMI on Log-transformed PK parameters ($AUC_{0\text{-last}}$, $C_{max}$ and $\lambda_z$).** The P value determines if the estimated slope of the fitted line (equation given) is significantly different to 0. Significant values below an alpha of 0.05 are in red.

### 5.3.4. Impact of genetic variants on INH PK parameters

CES1 SNP genotypes were also tested as single factors to assess their effect on PK parameters using general linear models.

The results listed in Table 5-5 indicate that rs3815583 SNP has a significant effect on INH elimination (P=0.019). As shown in Figure 5-3 elimination rates were lower in patients carrying rs3815583 minor allele (G).

**Table 5-5 Effect of CES1 SNPs on PK parameters. Beta indicates effect size.**

| Variables | $AUC_{0\text{-last}}$ | | $C_{max}$ | | $\lambda_z$ | |
|---|---|---|---|---|---|---|
| | **Beta [95% CI]** | **P value** | **Beta [95% CI]** | **P value** | **Beta [95% CI]** | **P value** |
| rs3815583 | -0.194 [-0.442/0.053] | 0.122 | -0.041 [-0.242/0.160] | 0.684 | 0.235 [0.040/0.431] | 0.019 |
| rs62028647 | 0.050 [-0.197/0.296] | 0.689 | 0.0241 [-0.174/0.222] | 0.809 | 0.118 [-0.079/0.315] | 0.237 |
| rs2244613 | -0.084 [-0.327/0.160] | 0.496 | -0.046 [-0.241/0.149] | 0.641 | -0.048 [-0.244/0.148] | 0.626 |

P-values highlighted in red are statistically significant to an alpha value of 0.05

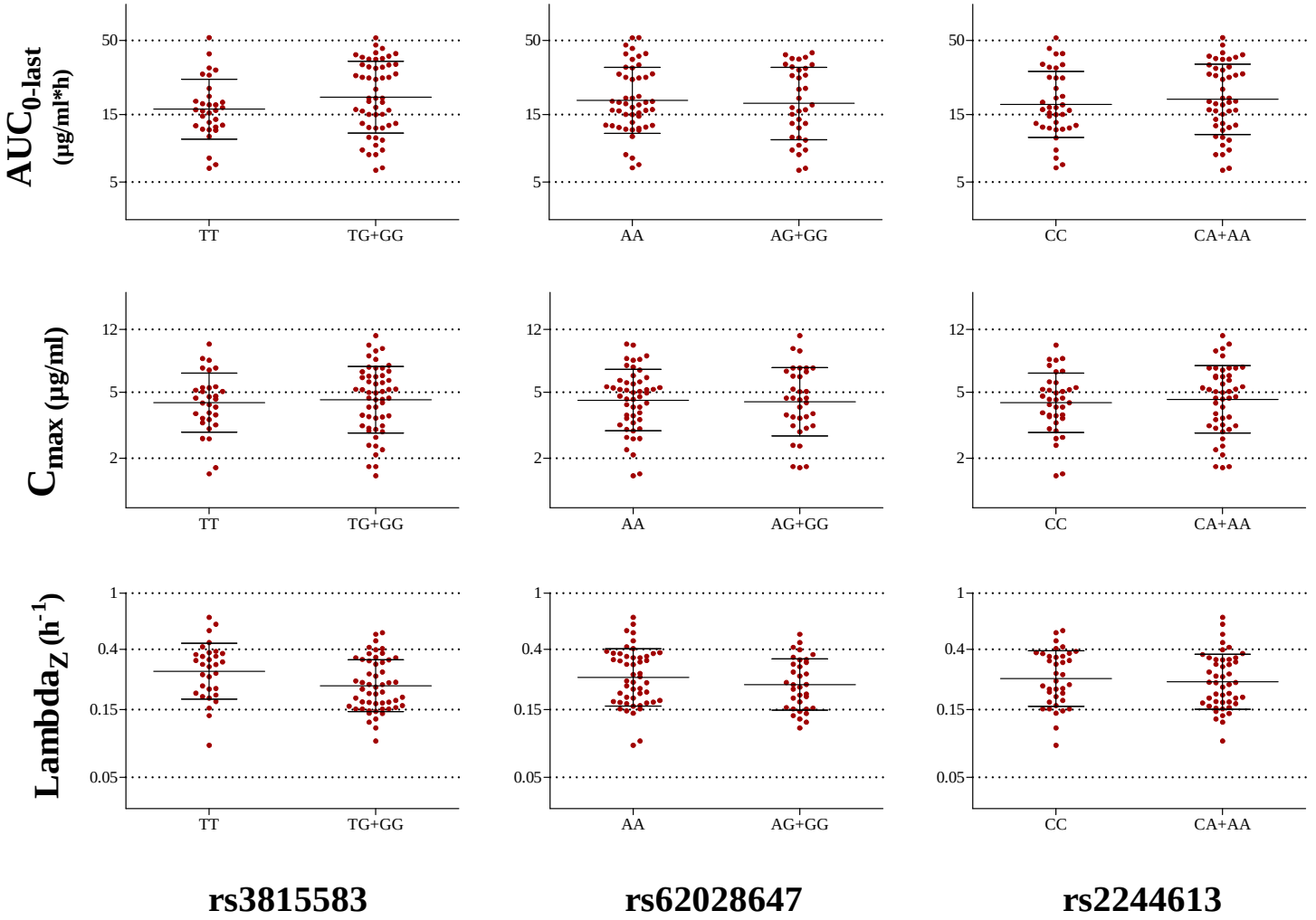


**Figure 5-3 Impact of CES1 genetic variants on PK parameters ($AUC_{0\text{-last}}$, $C_{max}$, and $\lambda_z$).** The lines and bars represent the mean and standard deviation (SD). Y axis is displayed in a back transformed log-scale.

### 5.3.5. Multifactorial models and the effect of factor interactions on PK parameters

A general linear model analysis was used to generate a multifactorial model that best explained the variance of the response variables, in this case, INH PK parameters. The explanatory variables included HIV and TB infection status, rs3815583 genotype, as well as an interaction between HIV and rs3815583. Throughout the exploratory analyses no other interactions, either with TB or other SNPs, showed any relationship with PK parameters and therefore were excluded from the final analysis.

Corrected models included as covariates those explanatory variables that had been identified in the single analysis as strong modifiers of each particular PK parameter.

The results of the multi-factorial models are shown in Table 5-6. It shows the non-corrected and corrected models for each PK parameter.

Both TB (P=0.011) and HIV (P=0.031) infection had a significant effect on INH $AUC_{0\text{-last}}$. However, only HIV (P=0.010) remained a predictor of $AUC_{0\text{-last}}$ after correcting for BMI.

The interaction between HIV and rs3815583 is not quite significant but as shown in Figure 5-4 it appears that an increase in $AUC_{0\text{-last}}$ happens only when HIV positive individuals carry rs3815583 minor allele. This effect is also supported by statistical contrasts in the general linear model, for both the uncorrected and corrected models.

Interestingly, for $C_{max}$ in the non-corrected model, it is the interaction between HIV and rs3815583, but not the individual main effects, which was significant (P=0.038). The differences in $C_{max}$ between groups are shown in Figure 5-5. It appears that opposing effects occur for different alleles of rs3815583, with those patients infected by HIV having lower values of $C_{max}$ when wild-type (WT), and higher values of $C_{max}$ when carrying the G minor allele. After correcting for sex and BMI the interaction is not significant (P=0.051).

Finally, the overall main effects of HIV infection and rs3815583 were significant for INH elimination rate ($\lambda_z$) before and after correction for sex and adjusted INH dose. The HIV*rs3815583 interaction did not significantly affect INH elimination. Therefore, regardless of the other factor, patients infected with HIV and patients carrying a minor allele of rs3815583 had significantly lower elimination rates. The differences in $\lambda_z$ between groups can be found on Figure 5-6.

**Table 5-6 Multifactorial model results. Both not corrected and corrected for main demographic and clinical factors.**

| Model terms | $AUC_{0\text{-last}}$ | | $C_{max}$ | | $\lambda_z$ | |
|---|---|---|---|---|---|---|
| | No corrections | Corrected | No corrections | Corrected | No corrections | Corrected |
| **Sex** | N/A | - | N/A | 0.028 | N/A | 0.096 |
| **BMI** | N/A | 0.057 | N/A | 0.047 | N/A | - |
| **mg/kg** | N/A | - | N/A | - | N/A | 0.013 |
| **TB** | 0.011 | 0.160 | 0.050 | 0.369 | 0.908 | 0.808 |
| **HIV** | 0.031 | 0.010 | 0.820 | 0.324 | 0.018 | 0.013 |
| **rs3815583** | 0.117 | 0.308 | 0.733 | 0.851 | 0.018 | 0.046 |
| **HIV*rs3815583** | 0.068 | 0.137 | 0.038 | 0.051 | 0.376 | 0.196 |

Significant P values to an alpha of 0.05 are shown in red.

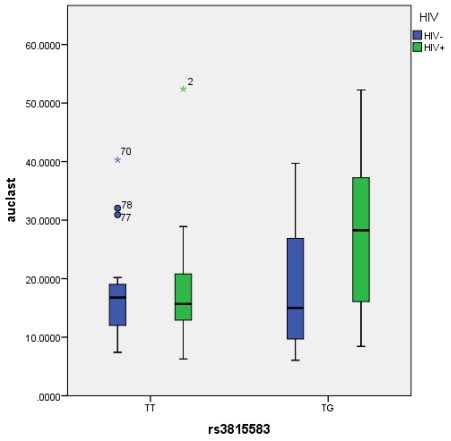


**Figure 5-4 $AUC_{t\text{-}last}$ (µg/ml*h) vs rs3815583 genotype in the presence and absence of HIV infection**

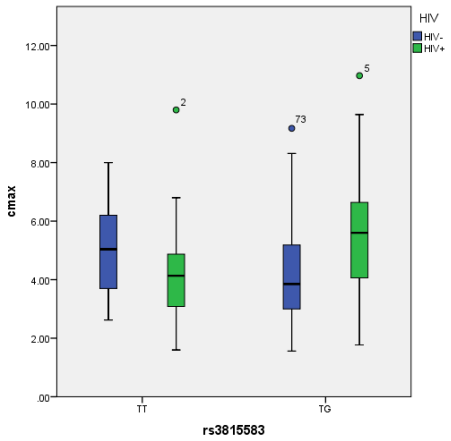


**Figure 5-5 $C_{max}$ (µg/ml) vs rs3815583 genotype in the presence and absence of HIV infection**

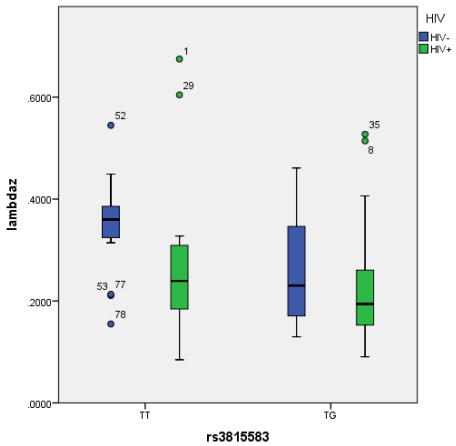


**Figure 5-6 $\lambda_z$ ($h^{-1}$) vs rs3815583 genotype in the presence and absence of HIV infection**

## 5.4. Discussion

In this study a series of SNPs located on CES1 gene were tested to help clarify an involvement of this enzyme in INH metabolism. We found evidence that rs3815583 minor allele carriers (G) achieved significantly higher $C_{max}$ and slower elimination rates ($\lambda_z$) than WTs when assessed independently, staying a significant predictor of the latter even in corrected multifactorial models.

The functional effect of rs3815583 SNP has not yet been clarified, therefore we can only speculate about its mechanism of action. Rs3815583 is located in the 5' untranslated region of the gene, which may suggest an effect on CES1 expression. The SNP has also been previously found significantly associated with be with appetite reduction in children treated with the CES1 specific substrate methylphenidate (Bruxel et al. 2012). However, it is possible that the influence may not be caused by a direct effect of rs3815583 but rather by a linkage to another functional SNP.

Yamada et al. (2010) had hypothesized that, as a result of their capacity to hydrolyse amide bonds as well as ester bonds, hepatic carboxylesterases could catalyse some of the reactions that INH undergoes during its hepatic clearance. In their study they evaluated CES1, CES2 and CES4 genetic variability in relationship to INH-induced hepatotoxicity. Their results showed that rs3815583 minor allele carriers displayed some trend towards significance in the context of hepatotoxicity as a side effect of INH treatment. Interestingly, the same SNP has been found to impact INH PK in the current study, especially when considered together with HIV infection.

The results show that 3 of the 6 SNPs tested appear to be monomorphic in this cohort, including rs71647871 (Gly143Glu). This is consistent with previous studies carried out in Asian populations, where its frequency appears to be even lower than in Caucasians (Suzaki et al. 2013).

Out of the three heteromorphic SNPs tested in this chapter, rs62028647 and rs3815583 displayed significant deviations from Hardy-Weinberg equilibrium. The reason for this was addressed in section 2.3.4. and relates to the assay's inability to discriminate homozygous minor allele carriers from heterozygous due to the existence of the CES1A3 pseudogene that displays a high degree of homology with CES1A1 gene.

This issue has been described repeatedly in the literature (Yamada et al. 2010, Zhu et al. 2012). For our analysis we assumed that both homozygous mutants and heterozygous were included in the group reported as heterozygous, and refer to them as minor allele carriers. Despite the difficulties for discrimination, the minor allele frequencies (MAF) found in this population were similar to those reported in Asian populations (dbSNP).

Neither rs2244613 nor rs62028647 exerted any noticeable effect on INH PK. Nonetheless, they displayed a similar allelic distribution profile which is consistent with the findings in Chapter 3 that indicated that both SNPs were in linkage disequilibrium. However, rs2244613 was found to be significantly correlated with clopidogrel-induced platelet aggregation and it was suspected to be a gain-of-function variant.

Perhaps the reason why rs2244613 and rs3815583 have not yet been found relevant in the same report is because the direction of their effect on CES1 activity is opposed. Unfortunately, rs3815583 could not be assessed in our clopidogrel population in Chapter 3 because it did not pass the quality controls.

The evidence generated in this chapter also indicates that HIV infection has a significant effect on INH PK, especially on $AUC_{0\text{-last}}$ and $\lambda_z$. Several studies have attempted to find a relationship between HIV and INH PK, mainly in children, but no significant change in INH PK parameters was found (McIlleron et al. 2009, Thee et al. 2011). McIlleron et al. did report lower INH exposure in males and in low body weight, which is consistent with our findings. We found in both univariate and multifactorial models significant lower $C_{max}$ values in males and there is a significant positive correlation between BMI and $C_{max}$. McIlleron et al. also found a significant difference between slow, intermediate and fast acetylators according to NAT2 genotype. NAT2 has been consistently reported as the main predictor of INH PK and the absence of it in our study is one of its limitations.

Another of the limitations of this study is that it is not truly relatable to real clinical practice. HIV therapy, that would normally interact with anti-TB drugs, was not allowed during the study. Moreover, as a single course of therapy, any long-term induction by RIF would not yet be apparent as induction generally displays delayed presentation. On the other hand, the absence of HIV treatment allows us to observe the isolated effects of HIV infection on INH PK parameters. Whether or not RIF has any effect on CES1 expression will be explored in Chapter 6.

The results reported in this chapter support the hypothesis that CES1 may play a role in INH metabolism. Such an idea is not surprising given the remarkable hydrolytic capacity of CES1, its promiscuity, and the potential of INH structure to be cleaved by it. Moreover even if CES1 did not represent a valid candidate for genetic testing and prediction of INH PK or treatment outcome, it would not be the first time that studies on genetic variability reveal previously unknown biological mechanisms.

This is the first time that a relationship between HIV infection and CES1 has been reported. We can only speculate about the mechanism of this interaction which could involve intracellular cholesterol metabolism on which it is known that HIV virus relies for entry and replication. There is also evidence that the virus can alter host cell lipid metabolism (Heaton and Randall 2011) and it has been specifically shown that esterase expression was induced in monocytes upon HIV infection (Petit et al. 1987). On the other hand, CES1 participates in lipid metabolism and is involved in cholesterol metabolism in macrophages (Crow et al. 2008). Moreover, its expression has already been found to be stimulated upon viral infection with hepatitis C virus (Blais et al. 2010). Therefore it can be hypothesized that a CES1 genetic variant like rs3815583 may behave differently to the WT upon HIV stimulation and thus affect differently the rate at which CES1 hydrolyses chemical compounds, such as INH.

Further work would involve *in vitro* studies exploring CES1 expression in the context of HIV infection and functional studies to determine the mechanism of the rs3815583 SNP. Regarding INH metabolism, further *in vitro* and *in vivo* assessments are needed to explore CES1 involvement and to clarify whether genetic variability is a factor that could explain the differences found in HIV effects on INH PK.

# CHAPTER 6

# Impact of rifampicin, rifabutin and rifapentine on CES1 and CES2 expression

# Chapter 6: Impact of rifampicin, rifabutin and rifapentine on CES1 and CES2 expression

## 6.1. Introduction

Carboxylesterases protein levels and hydrolytic activity are subject to great inter-individual variability but are also highly correlated with each other (Shi et al. 2011). Moreover, expression varies along developmental phases with a big surge in the early months of life and a steady increase during the infant and youth periods (Zhu et al. 2009).

Pathological conditions can affect expression of human carboxylesterases. A diminished expression of carboxylesterase 1 (CES1) and carboxylesterase 2 (CES2) has been reported in the presence of interleukin-6 (IL-6) (Yang et al. 2007) and the bacterial endotoxin lipopolysaccharide (LPS) (Mao et al. 2011). On the other hand, increased CES1 expression was reported in *in vitro* hepatitis C infected cells (Blais et al. 2010).

CES1 gene expression can also be modified by exogenous compounds, including therapeutic agents. Some compounds, like phenobarbital (PHE), dexamethasone (DEX) or rifampicin (RIF), have demonstrated a remarkable capacity to modify expression of metabolizing enzymes and transporters in different tissues.

PHE appears to increase expression of carboxylesterases in humans and rodents (Zhu et al. 2000), and it has been shown to display variable inducibility potential depending on subject age (Xiao et al. 2012).

DEX has been reported to be a strong inducer of CES1 and CES2 expression in human hepatocytes. By contrast, in rats it was found to suppress hydrolytic activity by esterases (Zhu et al. 2000).

RIF is a first line antituberculosis agent and a well-established inducer of proteins involved in drug metabolism, such as cytochrome P450 (CYP P450), phase II metabolising enzymes and phase III transporters (Niemi et al. 2003). Its inductive mechanism has been extensively studied and is known to involve pregnane X receptor (PXR) (Goodwin et al. 2002).

The evidence available about RIF-mediated regulation of carboxylesterases in human liver is far from exhaustive and conflicting data exist. While some reports claim a moderate induction of CES1 and CES2 by RIF (Zhu et al. 2000, Yang and Yan 2007) others have failed to reproduce these results (Nishimura et al. 2004).

The cell signalling for CES1 and CES2 expression has been explored by some groups. An involvement of PXR and glucocorticoid receptor (GR) was suggested by Zhu et al. (2000) when similar expression profiles to those of CYP3A4 arose in human primary hepatocytes. CYP3A4 dose-dependent regulation via these nuclear receptors has been described (Pascussi et al. 2001, Novotna and Dvorak 2014). Further evidence for PXR involvement in CES2 regulation was reported by Yang and Yan (2007). It has also been suggested that the p38MAPK-NF-κB pathway

controlled the suppression of CES1 and CES2 in the presence of LPS (Mao et al. 2011). Moreover, the Nrf2 (nuclear factor-like 2) antioxidant stress response pathway was found to control the inducible regulation of CES1 through binding to the antioxidant response element (ARE) located in the 5'-flanking region (Maruichi et al. 2010).

Gene expression of hepatic enzymes varies to meet the body metabolic demands and can be explored by exposing primary hepatocytes to therapeutic agents *in vitro* (Williamson et al. 2013). For protein-coding genes the intracellular levels of messenger RNA (mRNA) can provide a measure of gene transcription rates.

In order to expand our current knowledge on carboxylesterases regulation, the impact of the antituberculosis agents RIF, rifabutin (RBT) and rifapentine (RPT), prototypical gene inducers, on CES1 and CES2 gene expression were assessed *in vitro* by measuring mRNA intracellular levels in human primary hepatocytes.

## 6.2. Methods

### 6.2.1. Primary hepatocytes incubation and mRNA isolation

Thawing, plating, maintenance and treatment of cryopreserved primary human hepatocytes (Life Technologies, Paisley, UK) from 5 donors as well as mRNA isolation and cDNA (coding DNA) synthesis by reverse transcription polymerase chain reaction (RT-PCR) are described in detail by Williamson (2013). Donor demographics can be found in Table 6-1.

Briefly, cryopreserved hepatocytes were thawed at 37ºC for about 2 minutes before addition of cryopreserved hepatocyte recovery medium (CHRM®) (Life Technologies, Paisley, UK) and centrifugation. Hepatocytes were then resuspended in Williams E media (Life Technologies, Paisley, UK) with Phenol red (500 ml) and chemical and nutritional supplements. Cell viability was calculated by trypan blue exclusion. Cells were planted in 24-well plates at a density of $2 \times 10^5$ cells/well and incubated for 24 hours at 37ºC. Media was then replaced with treatment media containing RIF, RBT and RPT at 0.5, 5, 10, and 50 µM and incubation continued for another 24 hours. The experiment was carried out in triplicate. mRNA was extracted using a standard method recommended by Life Technologies involving lysis with Trizol® reagent (Life Technologies, Paisley, UK). Reverse transcription of mRNA to cDNA was carried out on a GeneAmp PCR 9700 (Applied Biosystems, Foster City, California, USA) using a Taqman® reverse transcription assay. DNA purity was assessed by the $A_{260}/A_{280}$ absorbance ratio. cDNA samples were diluted with PCR grade water (Sigma-Aldrich, St Louis, Missouri, USA) and normalized to a final concentration of 20 ng/µl (Williamson 2013).

**Table 6-1 Human donor demographics of the cryopreserved hepatocytes used in the experiment.**

| Donor | Viability (%) | Sex | Age (years) | Race |
|---|---|---|---|---|
| Donor 1 | 95 | Male | 68 | Caucasian |
| Donor 2 | 94 | Female | 47 | Indian |
| Donor 3 | 96 | Female | 36 | Caucasian |
| Donor 4 | 95 | Female | 72 | African American |
| Donor 5 | 94 | Female | 12 | Caucasian |

### 6.2.2. CES1 and CES2 mRNA quantitation

Taqman® gene expression assays for CES1 (Hs00275607_m1) and CES2 (Hs01077945_m1) as well as TaqMan® gene expression Master Mix were purchased from Applied Biosystems (Applied Biosystems, Foster City, California, USA). Amplification was performed in an Opticon2 Fluorescence Detector (MJ Research, Bio-Rad, Hertfordshire, UK). The amplification reaction was carried out in white 96-well plates. Each well contained a total reaction volume of 25 µl. The reaction consisted of 12.5 µl of TaqMan® gene expression Master Mix, 1.25 µl of Taqman® gene expression reagent containing the fluorescent probe, 9.25 µl of PCR grade water (Sigma-Aldrich, St Louis, Missouri, USA) and 2 µl of normalized sample (20 ng/µl). Cycle conditions were 95ºC for 15 minutes followed by 50 cycles of 95ºC for 15 seconds and 60ºC for 1 minute.

Each control and treatment condition was performed in triplicate for all three compounds and gene expression analysis was carried out twice on each cDNA sample obtained, with a final number of 6 Ct values per control and treatment condition.

Gene expression data is often presented as a relative measure compared to an internal reference, a gene whose expression is unaffected by the treatment at test. For this report, the gene selected as internal control was glyceraldehyde-3-phosphate dehydrogenase (GAPDH).

### 6.2.3. Data analysis

Data were analysed by the comparative $C_t$ method, a widely used method to present gene expression data that compares expression between a control and the testing conditions, having both been previously normalized to a reference gene, or housekeeping gene, in this case GAPDH.

To achieve that, first we obtained the difference between $C_t$ values of the target gene and the reference gene ($\Delta$ $C_t$) and then the difference between $\Delta C_t$ of the test and control conditions ($\Delta\Delta$ $C_t$). Relative gene expression is given by the equation: $2^{-\Delta\Delta Ct}$. A fully developed explanation of the equation has been described in the literature (Livak and Schmittgen 2001). The results are ultimately expressed as the fold change in gene expression of the test conditions in relationship to a control (0 µM). Mean fold change in expression was visually analyzed for individual donors. Statistical analysis was performed on grouped data only.

All statistical analysis and figures were produced using Graphpad Prism v. 5.01 (La Jolla, California, USA). Normality of the data was tested using Shapiro-Wilk test. Significant differences in gene expression between the different concentrations and the untreated controls were assessed using paired t-test or Wilcoxon signed-rank test for normally and non-normally distributed data, respectively. P values to an alpha of less than 0.05 were considered statistically significant.

Statistical analysis was performed on grouped data. Individual donor mean CES1 and CES2 fold change in expression is also shown.

## 6.3. Results

Amplification plots for each of the data points were assessed visually after gene expression q-PCR to confirm adequate amplification and ensure that the amplification plot crossed the threshold during the exponential phase of the curve. Samples that failed to amplify were excluded from the analysis.

$C_t$ values obtained from both genes were normalized against the internal reference gene, GAPDH. The $C_t$ comparative method was used to assess relative gene expression of CES1 and CES2. Changes in expression between the different concentrations of RIF, RBT and RPT were then assessed by comparing to the untreated control.

A Shapiro-Wilk test for normality was carried out on all the data and a t-test or Wilcoxon signed-ranked test was performed to explore significant differences in expression between the treated samples and untreated controls. Mean fold changes, standard deviation, median and range of each of the treatment conditions compared to untreated controls for both genes can be found in Table 6-2. A visual representation in the form of box and whiskers can be found in Figure 6-1. Values that were found to be significantly different to the untreated control ($P<0.05$) are marked with a red asterisk. Individual donor mean fold changes in CES1 and CES2 expression can be found in Figure 6-2.

As expected when using human primary hepatocytes, substantial variability in gene expression was observed between donors. However, as shown in Figure 6-1, RIF appears to exert a slight suppression of both CES1 and CES2 gene expression with significant P values for CES2. Both genes, in fact, follow a similar trend but the difference in CES1 expression did not reach significance.

There was no evidence of major changes in expression of CES1 and CES2 in samples treated with RBT and RPT, although some of the values at the higher concentrations (10 and 50 µM) were found to be significantly different to the control.

When observing individual donors gene expression data, 3 out of 5 and 4 out of 5 donors display a slight suppression of CES1 and CES2 respectively in the presence of RIF (Figure 6-2). Donor 3 does not show this trend with RIF but appears to be slightly supressed by RBT instead. For donors 1 and 4 certain induction is apparent at higher concentrations of RBT (10 µM and 50 µM). No consistent pattern appears for RPT treated hepatocytes in either gene.

**Table 6-2 Change in relative gene expression of CES1 and CES2 across a concentration range of RIF, RBT and RPT when compared to control. Mean fold change in gene expression of CES1 and CES2 when treated with concentrations from 0.5 to 50 µM of RIF, RBT and RPT compared to the control that was not treated with any drug. The table shows the mean, standard deviation (SD), median, range of values and P value (considered a significant change when P<0.05) obtained by paired t-test or Wilcoxon signed-rank test.**

| Compound | Conc. (µM) | CES1 fold gene expression | | | | | CES2 fold gene expression | | | | |
|---|---|---|---|---|---|---|---|---|---|---|---|
| | | Mean | SD | Median | Range | P value | Mean | SD | Median | Range | P value |
| **RIF** | **0.5** | 1.15 | 1.81 | 0.64 | 0.15-8.46 | 0.0878 | 0.57 | 0.43 | 0.46 | 0.06-1.59 | 0.0088 |
| | **5** | 0.89 | 1.06 | 0.42 | 0.04-3.95 | 0.2343 | 0.58 | 0.59 | 0.44 | 0.07-2.46 | 0.0129 |
| | **10** | 1.02 | 1.37 | 0.43 | 0.07-5.10 | 0.4237 | 0.52 | 0.54 | 0.32 | 0.03-2.31 | 0.0021 |
| | **50** | 1.05 | 1.06 | 0.66 | 0.09-4.17 | 0.6406 | 0.93 | 0.86 | 0.72 | 0.05-3.84 | 0.3107 |
| **RBT** | **0.5** | 1.32 | 1.14 | 1.10 | 0.09-4.2 | 0.1388 | 1.52 | 2.35 | 0.80 | 0.29-10.63 | 0.3194 |
| | **5** | 0.92 | 0.75 | 0.64 | 0.08-3.61 | 0.9455 | 1.12 | 1.54 | 0.63 | 0.14-7.84 | 0.2133 |
| | **10** | 2.00 | 2.63 | 0.54 | 0.08-9.78 | 0.2319 | 1.70 | 1.33 | 1.38 | 0.05-4.96 | 0.0602 |
| | **50** | 4.52 | 10.03 | 1.50 | 0.07-48.5 | 0.0085 | 1.78 | 1.81 | 1.19 | 0.13-8.17 | 0.1275 |
| **RPT** | **0.5** | 1.23 | 1.50 | 0.89 | 0.02-7.84 | 0.8554 | 4.07 | 12.29 | 0.95 | 0.11-65.8 | 0.1243 |
| | **5** | 1.21 | 0.78 | 1.08 | 0.31-3.58 | 0.1697 | 1.28 | 0.87 | 1.25 | 0.03-3.03 | 0.322 |
| | **10** | 2.24 | 3.57 | 1.27 | 0.02-17.15 | 0.0703 | 1.87 | 1.55 | 1.59 | 0.13-7.11 | 0.0232 |
| | **50** | 1.92 | 2.06 | 1.17 | 0.08-9.65 | 0.0225 | 1.35 | 1.72 | 0.63 | 0.04-5.82 | 0.6968 |

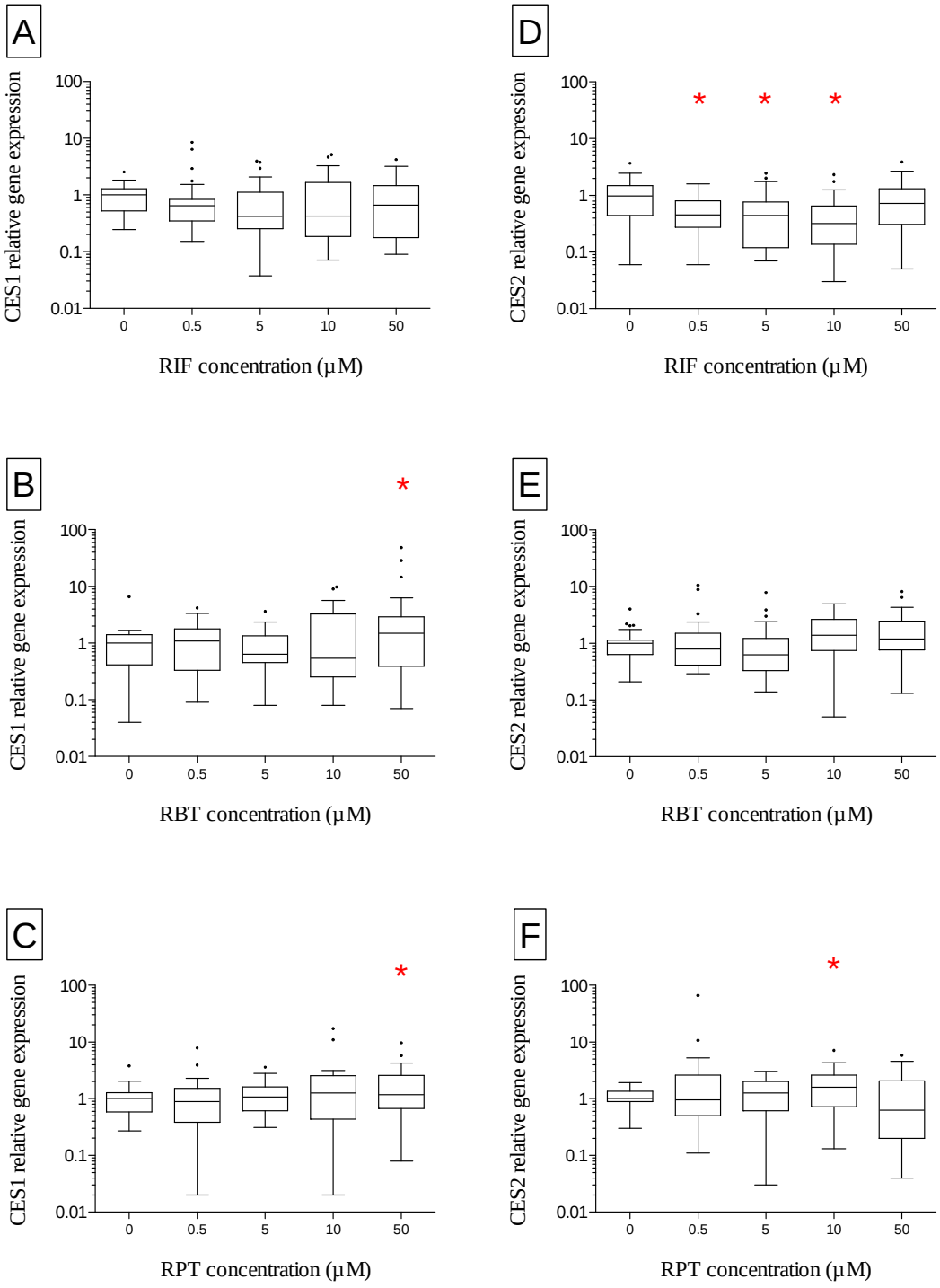


**Figure 6-1 Box and whiskers plots for relative gene expression of CES1 and CES2 genes.** A-C plots show the relative gene expression of CES1 gene after treatment with 0 to 50 µM concentrations of RIF, RBT and RPT, respectively. D-F plots show the relative gene expression of CES2 gene after treatment with 0 to 50 µM concentrations of RIF, RBT and RPT, respectively. Data is plotted as Tukey boxes representing the median and interquartile range (IQR), outliers are values further than 1.5 IQR above or below the box. Results found to be significantly different from the untreated control are marked with a red asterisk.

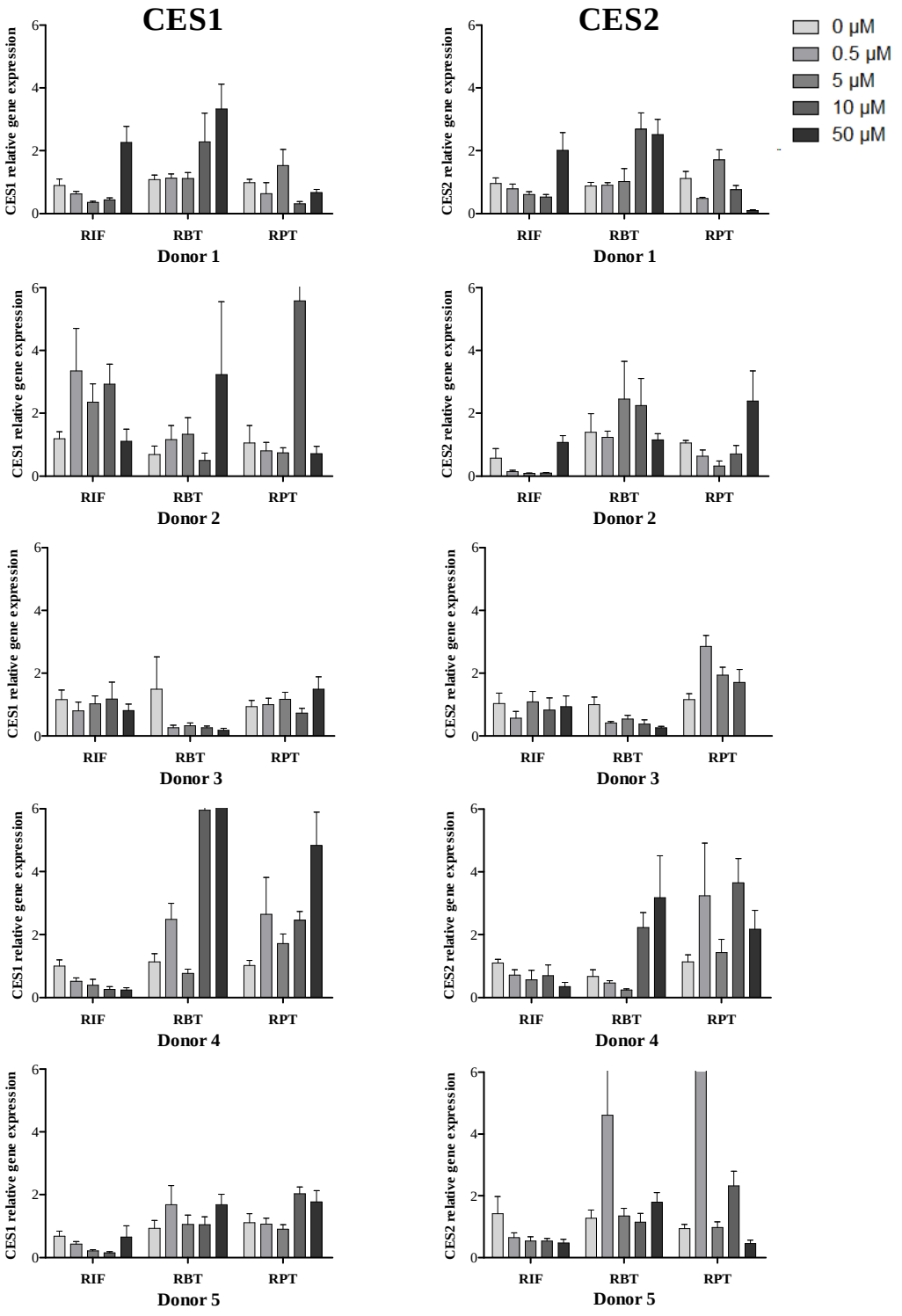


**Figure 6-2 Mean fold change in CES1 and CES2 expression per human donor.** Effects on CES1 and CES2 mRNA expression in primary cultures of human hepatocytes after exposure to RIF, RFB and RPT at concentrations ranging from 0 to 50 µM for 24 hours. Data are expressed as the mean fold change in target to GAPDH mRNA. Experiments were performed in triplicate. Data are shown as mean ± SEM (standard error of the mean).

## 6.4. Discussion

The work described in this chapter aimed to improve our understanding of the drug-induced regulation of CES1 and CES2 in humans. Our findings suggest that CES1 and CES2 genes do not undergo induction in the presence of rifamycins.

There is an extensive body of literature on RIF involvement in drug-drug interactions (DDIs) mediated by its capacity to interact with the intracellular machinery and translocate to the nucleus to modify transcription of genes encoding proteins involved in xenobiotic transport and metabolism. However, little is known about the intracellular players on either basal or inducible regulation of carboxylesterases.

It has been suggested that CES1 and CES2 may be regulated by the same pathway as CYP3A4 because of a similar expression profile displayed in an experiment with cultured human hepatocytes from 3 human donors at a single RIF concentration, 10 µM (Zhu et al. 2000). On a deeper exploration Yang and Yan (2007) observed the effect of knockdown and overexpression of PXR in the presence of RIF (25 µM) and 8-methoxypsoralen on both CYP3A4 and CES2. They showed that in fact, a similar pattern arose for both enzymes: a decrease in protein levels in PXR knockout cells and an increase in PXR overexpressors. However, Nishimura et al. (2004) reported that CES1 mRNA was not affected by exposure to RIF (50 µM) in human primary hepatocytes.

In the work described in this chapter there does not seem to be any remarkable induction of RIF, RBT or RPT on the expression of either CES1 or CES2 across concentrations. If anything, one could observe a slight suppression of both genes in the presence of increasing concentrations of RIF, but so modest that no clinical repercussion would be expected.

In this study then, the previously observed parallel pattern between CYP3A4 and CES1/CES2 was not reproduced; CYP3A4 gene expression had been previously reported for this cohort and showed induction rates of up to 100-fold (Williamson et al. 2013). This discrepancy with previously reported results may be due to regulation of CES1 and CES2 taking place via an alternative intracellular pathway, maybe in combination with PXR and GR nuclear receptors; or to experimental conditions, optimally CYP3A4 and CES1/CES2 mRNA levels should be assessed in parallel.

The use of primary hepatocytes to evaluate drug-mediated modifications of protein expression also carries some intrinsic limitations like differences in mRNA expression and activity of some enzymes compared to *in vivo* conditions, and the high inherent inter-donor variability in protein expression at basal levels and in response to regulation. High numbers of donor samples would be necessary for clearer results, however human tissue resources are scarce and expensive.

Other limitations of this study are the absence of data on the behaviour of intermediate proteins that make up the regulatory cascades, which would offer a more complete picture of the mechanisms underlying expression patterns.

Also, a wider range of concentrations could have potentially informed us better of a biphasic effect or the involvement of different nuclear receptors at different concentrations as has been previously found for CYP3A4 in the presence of DEX. The latter appears to be regulated by GR at nanomolar concentrations and by PXR at micromolar concentrations (Pascussi et al. 2001).

The biggest mean fold change reported in this study was 4.52; such a change at the molecular level is unlikely to have a clinically relevant effect. These data can, however, help shed some light on the mechanisms of regulation of carboxylesterases for which little is yet known. For instance, the similarity in expression patterns between CES1 and CES2 may indicate shared regulatory pathways.

There is certainly a need to increase our understanding of this topic because the evidence available is very limited. Future work should include a more exhaustive exploration of the intracellular pathways involved in the surge of expression during developmental stages, the main controllers of basal and inducible expression of CES1/CES2 as well as determinants of inter-individual variability. In addition, it is important to define the role played on gene expression by genetic variability in CES1/CES2 genes, regulatory pathways proteins, or membrane transporters that determine intra-hepatocyte concentrations. Moreover, quantification of the magnitude of induction or suppression would help explore clinically relevant DDIs.

Finally, inter-species differential patterns of expression exist. For instance, in rodents carboxylesterase is present in plasma but in humans it is not (Li et al. 2005). It has also been reported that there is a difference in DEX-dependent inducibility, with increased carboxylesterase expression in humans but reduced expression in rats.

Unravelling species differences in drug metabolism and gene expression will reduce uncertainty when using rodent models in the pre-clinical stages of drug development allowing for safer therapies and less unexpected side effects in post-commercialization stages.

In conclusion, CES1 and CES2 hydrolyse a high number of therapeutic agents with ester and amide bonds in their structure, and the list grows every year, as esterification of active compounds to increase intestinal absorption is still a popular strategy in drug development. Improving our understanding of the regulation of expression and activity of this family of enzymes as well as the identification of the main factors that affect them is important to identify potentially harmful DDIs. In particular, knowledge about the effect of RIF and other rifamycins on CES1 and CES2 gene expression would help us predict if a DDI is or not expected between the antituberculosis treatments and CES1/CES2 substrates.

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# CHAPTER 7

# Final discussion

# Chapter 7: Final discussion

Carboxylesterase 1 (CES1) is the main human liver esterase and is involved in the metabolism and disposition of numerous endogenous and pharmacological compounds. Understanding the factors that govern CES1 activity can clarify some of the variability found in response to treatments with some of the drugs metabolized by this enzyme. Unfortunately, there is still much uncertainty about the factors controlling CES1 expression and activity.

In the course of this thesis some of the many factors that affect CES1 hydrolysing capacity have been evaluated, such as genetic variability, drug-drug interactions (DDIs), protein expression, subject demographics and pathological conditions (See Figure 7-1)

Concerning genetic variability in the CES1 gene, there are currently 1030 CES1 genetic variants listed in the dbSNP database (http://www.ncbi.nlm.nih.gov/snp Last accessed Sept2014). However, only a small fraction of them have been thoroughly characterized. During the work developed for this thesis 2 of the 6 variants explored arose as potential modifiers of pharmacological treatment: rs2244613 and rs3815583.

In Chapter 3 it was found that rs2244613 minor allele carriers had significantly higher platelet reactivity than wild-types treated with clopidogrel. This SNP had been previously found correlated with methylphenidate adverse events in a children cohort (Johnson et al. 2013), and with dabigatran plasma concentrations and its adverse effect of bleeding (Pare et al. 2013).

On the other hand, rs3815583 was shown in Chapter 5 to significantly affect INH pharmacokinetics, supporting previous studies that suggested CES1 involvement on INH-related hepatotoxicity (Yamada et al. 2010). Rs3815583 had also been linked to appetite reduction in children treated with methylphenidate (Bruxel et al. 2012).

One SNP previously found correlated with clopidogrel pharmacokinetics and platelet aggregation, rs71647871, was also assessed during this thesis, but no significant effect was found in either the clopidogrel (Chapter 3) or isoniazid (Chapter 5) populations due to its low frequency (Tarkiainen et al. 2012, Lewis et al. 2013, Zhu et al. 2013). Allele frequencies reported for this and other SNPs assessed in this thesis were consistent with what had been reported in the literature for Caucasian and Asian populations.

Linkage disequilibrium (LD) was explored between the SNPs assessed in this thesis and strong LD was observed between rs2244613 and rs62028647. Our data does not clarify whether the effects found for these SNPs during our studies are direct or the functional effect is due to another linked SNP. Further assessment is necessary to characterize the mechanism of action of these variants as well as clarify the actual perpetrators of the action.

Another of the factors that may affect CES1 activity is the occurrence of DDIs. In this thesis this aspect was explored by assessing clopidogrel pharmacokinetics in co-administration with non-nucleoside reverse transcriptase inhibitors (NNRTIs) *in vivo* (Chapter 4).

Our results do not indicate that NNRTIs would have a remarkable impact on clopidogrel pharmacokinetics, therefore not representing a source of harmful drug interactions. Some differences were identified between clopidogrel pharmacokinetic parameters in subjects treated with EFV and NVP. However, results of this small study should be considered carefully due to small sample size. Future trials comprising larger cohorts are desired to conclusively determine NNRTI impact on clopidogrel pharmacokinetics and anti-aggregation efficacy. Separate assessments should be carried out for EFV and NVP because if the differences hinted in our study were proved real this could influence clinicians' pharmacological choice in the treatment of HIV positive patients with heart conditions.

CES1 protein levels also affect drug hydrolysis rate and may impact treatment response. Gene expression levels are governed by intracellular pathways that may be altered in the presence of certain pharmacological compounds. In Chapter 6 CES1 and CES2 expression in the presence of prototypically PXR inducer rifampicin and two of its derivatives, rifabutin and rifapentine, was explored in primary hepatocytes.

Our results did not identify any inductive effect of any of these compounds on CES1 and CES2, suggesting that DDIs between rifamycins and CES1 substrates may not pose a risk. However, the pathways that control CES1 regulation of expression are poorly understood and more *in vitro* studies are necessary to characterize both basal and drug-induced regulation of expression of this enzyme.

Other factors that control CES1 expression and activity have been found in the course of this thesis. Women had been shown in some reports to display higher catalytic efficiency than men (Patrick et al. 2007) but the pattern was not always replicated (Zhu et al. 2009).

In Chapter 3 we find significantly higher concentrations of the direct product of CES1 catalytic reaction (CLPM) in females, supporting the idea of an increased esterase activity in this sex. This difference between sexes could, in the case of CLP, mean reduced treatment efficacy in females. However, platelet aggregation in our study cohort did not appear any different between sexes, making unlikely a difference in treatment outcome.

CES1 expression may also be modified by pathological conditions affecting individuals. In Chapter 5 of this thesis a relationship between CES1 and HIV infection was identified; shown as a differing response of isoniazid pharmacokinetic parameters for CES1 rs3815583 genotypes with and without infection.

Pursuing studies to functionally characterize the mechanism of this relationship could uncover biological processes involved in CES1 regulation or HIV pathogenicity that were not previously suspected. Moreover, this could also have other implications in drug metabolism not restricted to the field of TB pharmacokinetics.

In conclusion, all the above described determinants of CES1 expression and activity are factors that add up to the overall variability of CES1 hydrolytic capacity. CES1 is involved in numerous biochemical cellular processes, such as protein trafficking, lipid balance, and detoxification processes, which include disposition of pharmacological compounds. CES1 catalytic efficiency represents one of the many factors that govern drug pharmacokinetics and therefore affect treatment outcome or development of adverse events. Thus the more we understand about the factors that control CES1 activity and expression the closer we will get to design and deliver safe and efficacious treatments.

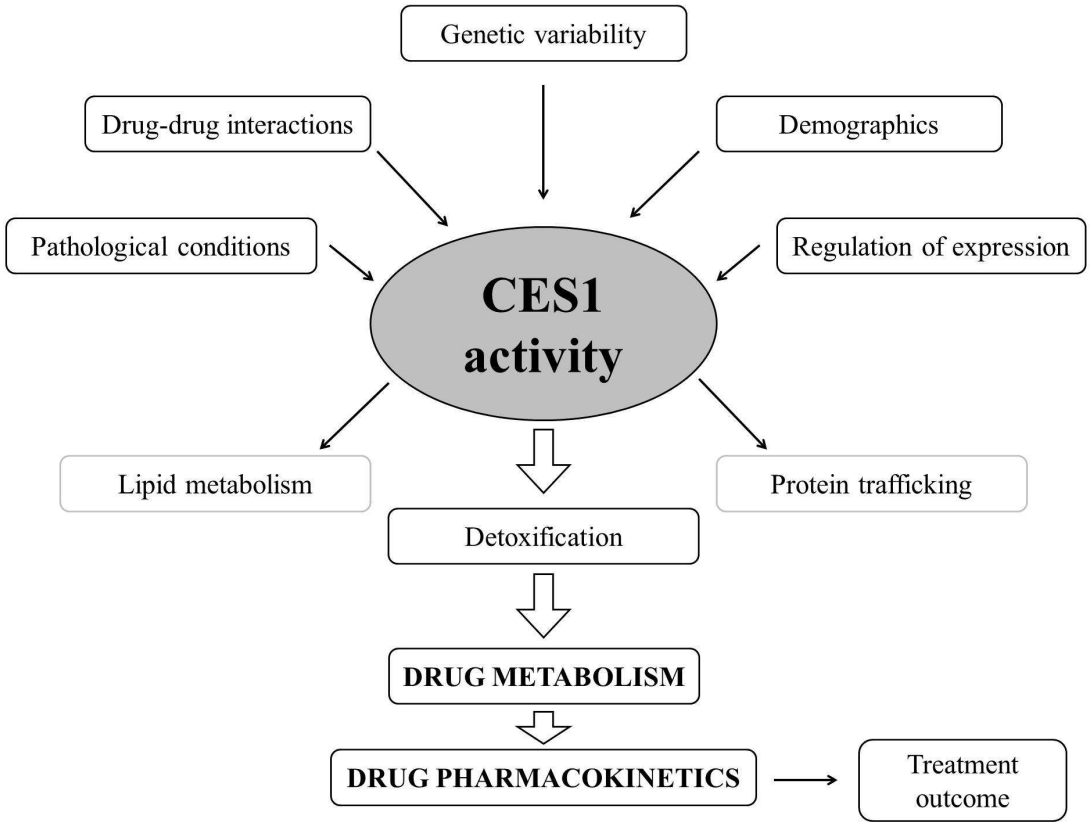


**Figure 7-1 Schematic diagram of CES1 activity.** CES1 carries out a diverse array of functions in human metabolism including processing of pharmacological compounds. CES1 catalytic efficiency is determined by a number of endogenous and exogenous factors, some of which were explored in the course of this thesis.

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# REFERENCES

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